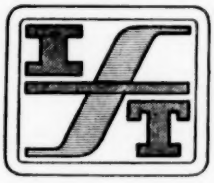
Food Research

An Official Publication of

The Institute of Food Technologists



IN THIS ISSUE

Thermal Death Time-Temperature Relationships of *Salmonella Typhimurium* in Chicken Muscle

Resistance of Bacterial Spores to Gamma Irradiation

Determination of the Solubility Index of Spray Dried Eggs

Antioxidant Activity and Antagonism to Microbial Growth by Piperine, Isonicotinyl Hydrazide and Related Compounds

Heat Resistance in Liquid Eggs of Some Strains of the Genus *Salmonella*

Effect of Sucrose and Ascorbic Acid on Quality Retention in Fresh and Frozen Strawberry Puree

The Diethylstilbestrol Content of Tissues of Treated Steers, Lambs, and Poultry

Effect of Aging and Cooking on the Distribution of Certain Amino Acids and Nitrogen in Beef Muscle

Detection of Washed Eggs by Conductivity Measurements. I. Test Methodology

Studies on the Use of Coating for Extension of Storage Life of Fresh Fajli Mango

A Histological and Histochemical Study of Beef Dehydration III. Influence of Pre-Cooking

Sclereid Development and the Texture of Bartlett Pears

Lysine Destruction in Casein-Glucose Interaction Measured by Quantitative Paper Chromatography

Heat Resistance of Strains of *Salmonella* in Liquid Whole Egg, Egg Yolk, and Egg White

Volume 19 JULY-AUGUST, 1954 Number 4

Food Research

An Official Publication of
The Institute of Food Technologists

Volume 19 JULY-AUGUST, 1954 Number 4

EDITORIAL BOARD

MARTIN S. PETERSON, *Editor-in-Chief*
(Editorial Office: 11606 South Bell Avenue, Chicago 43, Illinois)

J. GRANT ARMSTRONG
A. K. BALLS
J. S. BLAIR
KENNETH W. BRIGHTON
G. M. DACK
R. R. LEGAULT
JOHN R. MATCHETT
PAULINE PAUL
LOUIS B. ROCKLAND
ROBERT G. TISCHER
JAMES RICHARD VICKERY
O. B. WILLIAMS

FRED W. TANNER, *Editor Emeritus*
Z. I. KERTESZ, *Consulting Editor*

CHARLES S. LAWRENCE, *Business Manager*
176 West Adams Street
Chicago 3, Illinois

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 119-123 West Park Avenue, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

INSIDE SCIENCE

The Vital Story of VITAMIN C

by Science Writer

What does vitamin C do for human beings?

Medical research shows that it is essential in maintaining healthy bony tissue and for the formation of collagen which is the main supportive protein of skin, tendon, bone, cartilage and connective tissue. It is needed for vascular function and tissue respiration and in promoting the healing of wounds. In lactating women it has great importance in providing the infant with a sufficient amount of ascorbic acid to prevent scurvy, a disease to which very young babies are susceptible.

Much evidence is available in medical reports to show that a lack of this vital nutritive element can result in impaired health. And in those cases of frank scurvy which still occur, ascorbic acid is the specific medication.

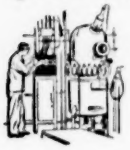
History. The history of vitamin C is fascinating. Scurvy was once the most prevalent disease in Europe. In the 18th Century the antiscorbutic value of fresh vegetables and citrus fruits was recognized.

Isolation. It was not until 1918-25 that almost pure ascorbic acid was concentrated from the lemon by Zilva and its important properties and molecular composition established. In 1927 he concluded that this so-called "reducing factor" and the antiscorbutic principle were closely related. In 1928, Szent-Györgyi, who started investigations in 1921, isolated a strong reducing compound from adrenal glands, oranges, cabbage, terming it "hexuronic acid."

Chemistry. The "reducing factor," "hexuronic acid," and vitamin C were all identified as the same in 1932 by various groups of workers. This was one year after C. G. King established the chemical identification of vitamin C.

Synthesis. In 1933 Reichstein and his colleagues in Switzerland successfully synthesized ascorbic acid. Almost simultaneously in England Haworth and associates accomplished the same. The Reichstein synthesis formed the basis for large scale commercial production by the world-famous firm of Hoffmann-La Roche.

Production. Following these discoveries came the establishment of manufacturing operations so that today daily production by the tons is the rule. The figures are really staggering. The amount produced weekly in the U. S. A. averages over 15 tons, equivalent to that contained in over 200 million oranges. It is remarkable that ascorbic acid can be produced synthetically and sold for only about $21. per kilogram. One kilo is sufficient to supply 92 people for one year with the minimum daily requirements set by the U. S. Food and Drug Administration.



Vitamin C made by the Roche process is identical chemically and in biological activity with Nature's own product. Because vitamins can be manufactured at a much lower cost than if they were extracted from natural sources they are widely used in dietary supplements.

Ascorbic acid is present in many foods. Nature supplies some with lavish amounts and stints on others. In fact, wide variations* have been found in the vitamin C content in varieties of the same fruit.

Deficiencies. Many surveys have shown widespread vitamin C deficiencies in the population. Physicians may call these subclinical, but they are deficiencies nevertheless.

Because of these situations—natural variation and widespread deficiencies—diet experts believe that it is desirable and in the public interest to standardize the vitamin C content of processed fruit and vegetable juices by the addition of enough pure crystalline ascorbic acid to make them consistently dependable sources of this necessary food element. Some of these juices are: tomato, grape, orange, grapefruit, pineapple.

Requirements. The U. S. Food & Drug Administration has set 30 milligrams as the adult minimum daily requirement of ascorbic acid. This daily intake is just about sufficient to prevent recognizable signs of vitamin C deficiencies although a subclinical or an unrecognized deficiency may exist. In view of this, the National Research Council has established 75 milligrams as the recommended daily male adult allowance and 70 mg. daily for female adults, which levels are supported by many controlled nutrition studies. However, the N. R. C. recommends 90 mg. daily for boys between 13 and 15, 100 mg. daily for boys between 16 and 20, and 150 mg. daily for lactating women.

This article is published in the interests of pharmaceutical manufacturers and food processors who make their good products better with essential, health-giving vitamin C. Reprints are freely available. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul Street, West; Montreal, Quebec.

*The table shows minimum and maximum levels of ascorbic acid in commercially canned juices. All figures are in milligrams per 100 grams of juice. Data from U. S. Department of Agriculture.

	Min.	*Max.*
Apple juice	0.2	3.6
Grape juice	0.0	4.7
Grapefruit juice	10.0	49.0
Orange juice	9.7	70.0
Pineapple juice	5.4	18.0
Tomato juice	2.5	32.0

Volume 19 JULY-AUGUST, 1954 Number 4

CONTENTS

THERMAL DEATH TIME-TEMPERATURE RELATIONSHIPS OF *Salmonella Typhimurium* IN CHICKEN MUSCLE. Dorothy L. Hussemann and Jo Kessel Buyske 351

RESISTANCE OF BACTERIAL SPORES TO GAMMA IRRADIATION. Bruce H. Morgan and James M. Reed 357

DETERMINATION OF THE SOLUBILITY INDEX OF SPRAY DRIED EGGS. S. J. Bishov and J. H. Mitchell, Jr. 367

ANTIOXIDANT ACTIVITY AND ANTAGONISM TO MICROBIAL GROWTH BY PIPERINE, ISONICOTINYL HYDRAZIDE AND RELATED COMPOUNDS. Torsten Hasselstrom, G. P. Dateo, H. S. Levinson, W. H. Stahl, E. J. Hewitt, and K. S. Konigsbacher . 373

HEAT RESISTANCE IN LIQUID EGGS OF SOME STRAINS OF THE GENUS *Salmonella*. A. Anellis, J. Lubas, and Morton M. Rayman 377

EFFECT OF SUCROSE AND ASCORBIC ACID ON QUALITY RETENTION IN FRESH AND FROZEN STRAWBERRY PUREE. D. G. Guadagni 396

THE DIETHYLSTILBESTROL CONTENT OF TISSUES OF TREATED STEERS, LAMBS, AND POULTRY. C. E. Swift 402

EFFECT OF AGING AND COOKING ON THE DISTRIBUTION OF CERTAIN AMINO ACIDS AND NITROGEN IN BEEF MUSCLE. Irene D. Ginger, James P. Wachter, D. M. Doty, B. S. Schweigert, F. J. Beard, J. C. Pierce, and O. G. Hankins . . . 410

DETECTION OF WASHED EGGS BY CONDUCTIVITY MEASUREMENTS. I. TEST METHODOLOGY. Raymond Hixon, R. J. Connolly, and George F. Stewart 417

STUDIES ON THE USE OF COATING FOR EXTENSION OF STORAGE LIFE OF FRESH FAJLI MANGO. A. N. Bose and G. Basu 424

A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF BEEF DEHYDRATION. III. INFLUENCE OF PRE-COOKING. Earl Auerbach, H. Wang, Virginia Bates, D. M. Doty, and H. R. Kraybill 429

SCLEREID DEVELOPMENT AND THE TEXTURE OF BARTLETT PEARS. Clarence Sterling 433

LYSINE DESTRUCTION IN CASEIN-GLUCOSE INTERACTION MEASURED BY QUANTITATIVE PAPER CHROMATOGRAPHY. A. R. Patton, R. C. Salander, and Marcus Piano . 444

HEAT RESISTANCE OF STRAINS OF *Salmonella* IN LIQUID WHOLE EGG, EGG YOLK, AND EGG WHITE. W. W. Osborne, R. P. Straka, and Hans Lineweaver 451

SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $5.50 per volume.

Non-members: Domestic, $6.50 per volume; foreign, $7.00 per volume.

Subscriptions and renewals are entered to begin with the first issue of the current volume.

Single copies of current volume: Domestic, $1.00; foreign, $1.15.

CORRESPONDENCE

Editorial: MARTIN S. PETERSON, Editor-in-Chief, 11606 South Bell Ave., Chicago 43, Ill.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: FOOD RESEARCH, 119-123 W. Park Ave., Champaign, Ill.

Members Subscriptions: THE INSTITUTE OF FOOD TECHNOLOGISTS, 176 W. Adams St., Chicago 3, Ill.

Advertising: Charles S. Lawrence, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in FOOD RESEARCH. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for FOOD RESEARCH should conform to the style used in the journal and be submitted in duplicate to the Editor-in-Chief. Authors of papers intended for FOOD RESEARCH should request from the Editorial Office a copy of the booklet "Information and suggestions for authors." FOOD RESEARCH reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

Copyright, 1954
By the Institute of Food Technologists
Printed in U. S. A.

Food Technology

Published Monthly by the

INSTITUTE OF FOOD TECHNOLOGISTS

The Companion Journal to

FOOD RESEARCH

If you are a reader of FOOD RESEARCH your library is incomplete without FOOD TECHNOLOGY—devoted to the publication of the results of research and their practical application in industry. Contributors to its pages represent many fields of scientific and technological interest.

SUBSCRIPTION RATES FOR 1955

FOOD RESEARCH	$8.50 Domestic	$10.00 Foreign
FOOD TECHNOLOGY . . .	8.50 Domestic	10.00 Foreign

Send Subscriptions to

FOOD TECHNOLOGY
119-123 West Park Avenue
CHAMPAIGN, ILLINOIS

An Authorized Binding

for

FOOD RESEARCH

Arrangements have been made by Institute of Food Technologists for subscribers to have their journals bound into distinctively designed books.

Six issues, January through December, bound in best grade brown washable buckram, imprinted with your name on cover, cost but $3.30 per volume.

Bound journals serve as an immediate reference for research and information. Properly displayed, they create a psychological impact, implying the time and effort spent to keep up-to-date on the most modern methods and materials.

Ship journals parcel post. Within thirty days after receipt, bound volumes will be shipped prepaid anywhere in the U. S. A. Full remittance must accompany order.

+ + +

Publishers' Authorized Bindery Service

(Binders of all Journals)

308 West Randolph Street **Chicago 6, Illinois**

NEW EDITION OF "INFORMATION AND SUGGESTIONS FOR AUTHORS" PUBLISHED

A new and expanded edition of the pamphlet "Information and suggestions for authors" has been published for those writing technical contributions for *Food Technology* and *Food Research*.

The pamphlet discusses the respective scopes of the two journals and the type of articles suitable for them. A detailed outline is presented of the customary organization of technical papers followed by statements concerning the preferred styles and methods of preparing manuscripts. A discussion of various details like units of measurements and their abbreviations, use of footnotes and literature citations, preparation of tables and figures, etc. follows.

In the following chapter the customary methods of correcting printer's proofs are described. Here a list is given indicating the more common corrections. Instructions for the ordering of reprints follow.

Prospective authors are urged to consult this pamphlet which will be sent free of charge upon a request addressed to the editorial office.

Authorship in *Food Technology* and *Food Research* is not restricted to members of the Institute of Food Technologists.

THERMAL DEATH TIME-TEMPERATURE RELATIONSHIPS OF *SALMONELLA TYPHIMURIUM* IN CHICKEN MUSCLE[a, b]

DOROTHY L. HUSSEMANN AND JO KESSEL BUYSKE
Department of Foods and Nutrition, School of Home Economics, University of Wisconsin, Madison, Wisconsin

(Manuscript received May 22, 1953)

In the literature dealing with Salmonella as a public health problem, there is a recurrence of the implication that cooked poultry serves as a source of infection for man. Browne *(2)* has made a summary of the reports of Salmonella food poisoning outbreaks which have occurred following the eating of chicken, goose, or turkey. The manner in which cooked poultry apparently causing food poisoning becomes contaminated with Salmonella, however, has not been clearly defined. Some evidence has accrued to suggest that Salmonella is present in market poultry. Schneider and Gunderson *(11)* found Salmonella on the skin of 4.4% of 1,014 chickens. Felsenfeld, Young and Yoshimura *(4)* reported that of chickens examined by them, 327 United States inspected and passed birds and 748 uninspected birds, or 0.9 and 10.8%, respectively, harbored Salmonella.

Assuming that viable Salmonella cells may occur on market poultry, the question of the role of cookery in protecting the consumer is of importance. The work here reported constitutes a further endeavor to determine the time-temperature relationships necessary for the destruction of Salmonella cells when present in or on chicken muscle.

PROCEDURE

Determination of the thermal death time of Salmonella in chicken muscle was attempted by mixing approximately known number of cells of *Salmonella typhimurium*, 84,[c] into ground samples of excised chicken muscle and heating the resultant mixture in glass tubes in a thermostatically controlled oil bath for a definite period of time. In preparing the cell suspension which served as inoculum, 24-hour cultures of *Salmonella typhimurium*, 84, grown on tryptone-glucose-extract (TGE) agar slants, were washed with sterile saline into sterilized dilution bottles containing glass beads and agitated for two minutes. The cell suspension was standardized by adjustment with sterile saline to a constant turbidity as measured on a photoelectric colorimeter. Plate counts of dilutions of the standardized cell suspension were made to determine the approximate numbers of viable cells present.

Preparation of chicken muscle for heat treatment. Samples of breast and leg muscle were taken from freshly-killed chickens as aseptically as possible. These were placed individually in sterile half-pint tared fruit jars and ground on a power blender. The samples were then weighed by difference and frozen. A small portion of each sample was plated on both SS and TGE agar in order to gain an approximate picture of the bacteriological flora of the chicken sample previous to freezing.

---

[a] Published with the permission of the Director of the Agricultural Experiment Station, Madison, Wisconsin.

[b] This study was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[c] This culture was obtained through the courtesy of Dr. Joshua Lederberg, Department of Genetics, University of Wisconsin.

For each series of thermal death time determinations, a sample of the previously prepared chicken muscle was removed from the freezer and allowed to thaw in the half-pint jar. Only those samples which had shown no growth on the selective SS agar, and little or no growth on the TGE agar in the earlier plating tests were used. The thawed sample was inoculated with 0.1 ml. of a standardized cell suspension of *Salmonella typhimurium*, 84, per g. of chicken muscle. The cell suspension contained approximately 36×10^6 cells per ml. The cells were thoroughly mixed into the ground chicken muscle with the power blender.

A stainless steel syringe, fitted with a brass tube instead of a needle, was used to place the inoculated chicken meat in sterile 10×125 mm. pyrex tubes. As soon as the tubes in a series had received their samples, they were sealed in the flame of a blast burner and placed in an ice-water bath.

Heat treatment of the tubes of inoculated chicken muscle. A constant-temperature oil bath containing cottonseed oil was used for the heat treatment. The thermal death time tubes were clamped to a brass rod which was suspended in the oil. All replicates for one time interval were clamped on to the same brass rod.

The tubes were then transferred from the ice-water bath to the oil bath. Four different heating temperatures were employed: 75°C., 80°C., 85°C., and 90°C. After a predetermined number of minutes, the tubes were removed from the oil bath, and immediately plunged into ice water.

Culturing of the heat-treated tubes of chicken muscle. Culturing the contents of the tubes which had been heated presented some difficulties, because, although the tubes could be opened, it was found impossible to draw the chicken muscle from the open tubes into a culture medium. After several preliminary trials, it was decided to break the processed tubes directly into the culture medium. Thus, the sealed tubes were taken from the ice water and placed in acetone to remove the cottonseed oil. The tubes were then rinsed in distilled water and put in 70% alcohol to sterilize the outside of the tubes. After 15 minutes in the alcohol, the tubes were rinsed 3 times in sterile water and then broken with sterile pliers into one-half pint fruit jars. These jars contained glass beads and 9 ml. of tetrathionate broth. The mixture was then shaken on the shaking machine for 2 minutes at room temperature. Clumps of chicken muscle which did not disintegrate in the shaking process were broken up with a sterile glass rod.

After the chicken meat was thoroughly dispersed in the tetrathionate broth, the jars were placed in a 37°C. incubator for 24 hours. One-tenth ml. samples of the incubated broth were plated on SS agar, and examinations were made for evidence of surviving *Salmonella typhimurium* organisms.

Determinations were also made by the use of copper-constantan thermocouples of the time necessary to bring the chicken muscle samples in the thermal death time tubes to the temperature of the oil.

Testing enrichment media. In carrying out these experiments some further consideration of the enrichment medium used seemed pertinent. The importance of an enrichment medium in obtaining maximum recovery of Salmonella from infected material has frequently been noted. In the earlier work in this laboratory, tetrathionate broth was selected for use in enrichment procedures. Workers in some laboratories [Browne *(2)*, Hinshaw and McNeil *(7)*, and Schneider *(10)*] have tended to favor the Selenite-F enrichment broth devised by Leifson *(9)* over tetrathionate broth for the isolation of Salmonella.

The quality most desired in the enrichment medium to be used in these experiments was that of making possible detection of viable Salmonella cells when present even in very low numbers. An attempt was made to determine the least number of Salmonella cells which appeared to survive and to multiply in tetrathionate broth and in Selenite-F broth. This was accomplished by preparing tubes containing 10 ml. of tetrathionate broth or Selenite-F broth in such a way that the calculated concentration of cells was approximately 1 cell per 10 ml., 1 cell per 100 ml., or 1 cell per 1,000 ml. of broth. By design some of the tubes did not contain viable cells, since the purpose of the experiment was to detect Salmonella when only very minimal numbers were present. Measured portions of the inoculated enrichment broths were plated after 24 hours of incubation at 37°C. All platings were in triplicate on both TGE and SS agar. After incubation at 37°C. all plates were searched for colonies of *Salmonella typhimurium*.

RESULTS

Data presented in Table 1 indicate that under conditions of these experiments *Salmonella typhimurium*, 84, in chicken muscle survived in 75% of the samples held at 75°C. for 5 or 10 minutes. After a 15-minute heating period at 75°C., 55% and after 20- and 40-minute heating periods, 45% of the samples still contained viable Salmonella. The percentage of positive samples for the 3-, 5-, 10-, 15-, 20-, and 40-minute periods at 80°C. were, respectively, 45, 41, 35, 29, 17, and 17. When the observations were carried on at 85°C., the percentage of samples containing viable Salmonella after heating for the same time intervals were 40, 38, 35, 33, 25, and 15. At 90°C. the percentage of plates showing growth of Salmonella after 3 minutes of heating was 25, after 5 minutes was 9, and after 10 minutes was 5%, and thereafter all plates appeared sterile. The lag time required to bring the chicken muscle samples to the designated temperatures varied from one and one-third to one and one-half minutes. The length of time that the samples were at a specific temperature was actually somewhat less, therefore, than that indicated.

TABLE 1

Percentage of chicken samples[1] containing viable Salmonella typhimurium after heat treatment[2]

Treatment	Time in minutes[3]					
	3	5	10	15	20	40
	%	%	%	%	%	%
75°C.	....	75	75	55	45	45
80°C.	45	41	35	29	17	17
85°C.	40	38	35	33	25	15
90°C.	25	9	5	0	0	0

[1] Sample inoculated with approximately 3.6×10^3 cells per g. of chicken muscle.
[2] This table represents a total of 622 replications. The average number of replications per time-temperature interval was 27 although there were as many as 56 trials per test period in some instances. In only one instance were there as few as 14 trials at any one time-temperature relationship.
[3] The time interval stated includes the lag time required to bring the sample to the indicated temperature.

Salmonella typhimurium, 84, appeared to survive under much more adverse time-temperature conditions in chicken meat than in other products which have been tested. Winter *et al. (14)*, Solowey, Sutton, and Calesnick *(13)*, Schneider *(10)*, and Ayres and Slosberg *(1)*, all reported apparent complete destruction of viable Salmonella in various egg substrates in 6 minutes or less at temperatures varying from 58.9°C. to 61.8°C. Gibbons, Fulton, and Reid *(5)* were unable to recover viable Salmonella in dried whole egg powder when the liquid whole egg was heated 25 minutes at 60°C. before drying. Gibbons and Moore *(6)* reported all viable Salmonella apparently destroyed in products baked at oven temperatures of 140°C. and 210°C. and in scrambled eggs and omelets reaching temperatures of 82°C. to 98°C. Cathcart, Merz, and Ryberg *(3)* stated that bringing custard to a second boil killed all Salmonella present, and that baking custard pies at 246°C. also destroyed the organisms. Solowey and Calesnick *(12)* found viable Salmonella present in food products which were heated 7 to 8 minutes at 69 to 72°C. They also reported Salmonella present in an egg scramble cooked 17 minutes and 11 seconds at 56°C.

In general, the destruction of Salmonella in egg products appeared to be effected at lower temperatures than those observed for one strain of Salmonella in the present study on chicken muscle. Obviously the substrates are quite unlike in nature, the egg products having a different pH, viscosity, and chemical composition than chicken muscle. Variances in these factors are known to affect the survival of bacteria during heat treatment, and may partially account for the fact that lower lethal temperatures were reported by workers studying survival of Salmonella in egg products.

The fact that *Salmonella typhimurium*, 84, in chicken muscle appeared to survive high temperatures and long heating periods when present in large numbers has tended to affirm the work of Hussemann and Wallace *(8)*. In their study chicken muscle was

inoculated with Salmonella by injecting large numbers of viable cells into the blood stream of the bird just previous to slaughter. They reported that although broiling and roasting the infected chickens reduced the numbers of organisms, no chicken was actually rendered free of viable Salmonella cells. During their cooking procedures, the breast and thigh muscles of the chickens reached temperatures of 85°C. to 98°C., yet Salmonella organisms were isolated from every cooked chicken.

The numbers of Salmonella present in the chicken muscle in the study of Hussemann and Wallace *(8)* and in the present study were probably greater than would be likely to occur in or on naturally infected chicken muscle. The use of massive numbers of cells as inocula in these experiments seems justified, however, since in examining other materials, such as chemicals, which may be incorporated into foods, relatively large amounts, providing for margins of safety, are frequently employed in the tests. The viable cell count for Salmonella occurring in naturally infected chicken appears not to have been reported. Studies attempting to survey market poultry for this factor are now in progress in this laboratory. It also is not known whether Salmonella isolated from chickens in the market are dispersed throughout the chicken muscle as an infection within the chicken, or whether the cells are present only on the surface of the chicken, possibly resulting from contamination during processing or handling. The present experiments, therefore, probably were conducted under much more severe conditions than would normally be encountered. It would seem, nevertheless, that the heat resistance of certain Salmonella in chicken may be greater than that generally assumed. One explanation of this observation may be that a protective effect of the chicken muscle is encountered, and therefore common temperatures of cooking do not destroy the organisms in chicken muscle, especially if the infection is a massive one. This problem appears significant in the light of reports that Salmonella can be isolated from market poultry.

In comparing the selenite broth with the tetrathionate broth for use as a medium for recovery of small numbers of surviving cells of *Salmonella typhimurium*, 84, it appeared that the tetrathionate broth was the more effective enrichment medium. More positive isolations were made using the tetrathionate broth than with the Selenite-F broth (Table 2). Furthermore, multiplication of the cells in the tetrathionate broth

TABLE 2

Comparison of two enrichment broths for detecting varying concentrations of cells of Salmonella typhimurium

Series	Enrichment broth	Approximate cell concentrations per ml. of broth	No. of replications	No. of isolations (incubation = 24 hr.)	
				TGE[1]	SS[1]
1	Tetrathionate	1:10	30	24[2]	24
	Selenite	1:10	30	22[2]	15
2	Tetrathionate	1:100	30	12	12
	Selenite	1:100	30	8	4
3	Tetrathionate	1:1000	30	6	5
	Selenite	1:1000	30	4	0

[1] TGE—Tryptone Glucose Extract Agar. SS—Salmonella-Shigella Agar.
[2] These isolations were made from a 10^6 dilution of the broth culture after incubation.
[3] These isolations were made from a 10^3 dilution of the broth culture after incubation.

appeared to be more rapid than in the selenite broth. Subsequent to an inoculation of approximately one cell per 10 ml. of broth, the average number of cells present in tetrathionate broth, as determined by plate count on TGE, after a 24-hour incubation period, was 39×10^6, while the average number present in the Selenite-F was 62×10^3 (Table 3).

These facts do not agree with the results of Browne *(2)*, and of Hinshaw and McNeil *(7)* who reported more positive isolations with the Selenite-F broth than with tetrathionate broth. Both Browne *(2)* and Hinshaw and McNeil *(7)* were doing isolation work and the number of cells present in their materials was not known. If many cells were present, this difference between the two enrichment media would not neces-

TABLE 3

Comparative rate of multiplication of Salmonella typhimurium in two enrichment broths when inoculated at an approximate concentration of 1 cell per 10 milliliters of broth

Enrichment broth	No. of replications	After 24-hour incubation period	
		TGE[1]	SS[1]
		cells/ml.	*cells/ml.*
Tetrathionate	30	39×10^6	22×10^6
Selenite	30	$62 \times 10^{3*}$	77×10^3

[1]TGE—Tryptone Glucose Extract Agar. SS—Salmonella-Shigella Agar.
*The true mean was probably somewhat larger than the number given, as four plates which were too crowded to count were not included in the calculation of the figure given.

sarily be obvious, and other factors may have determined the number of positive isolations. This present work seemed to indicate that with approximately equal number of cells present in the inoculum, and under conditions of pure culture and for the strain tested, the tetrathionate broth was the more effective enrichment medium. The effect of the presence of chicken muscle under these conditions was not tested.

SUMMARY

A study was made of the effect of heat on the survival of *Salmonella typhimurium*, 84, in chicken muscle. Samples of chicken muscle inoculated with large numbers of one strain of Salmonella were placed in thermal death time tubes. These were heated in an oil bath for varying time intervals at a series of temperatures which might be encountered during cookery and observations were made on the survival of the Salmonella.

Salmonella typhimurium, Strain 84, when present in chicken muscle appeared to survive higher temperatures than have been observed for destruction in other products. The conditions of these experiments were stringent, however, and it may not be possible to make direct application to the more natural situation. The data do appear to serve as a further warning that cookery may not serve to free poultry of certain of the Salmonella.

Addendum

Subsequent to the preparation of this paper a transplant of the strain of Salmonella employed in the experiments was sent to Dr. Hans Lineweaver, Head of the Poultry Products Division of the Western Utilization Research Branch. Under his direction, the strain was examined by Mr. W. W. Osborne, for heat resistance. Using 116,000 cells per TDT tube, and liquid egg as the suspending medium (pH 7.5), he described the organism as having "ordinary" heat resistance—2.8 minutes at 60°C. Appreciation is expressed for Dr. Lineweaver's and Mr. Osborne's co-operation.

LITERATURE CITED

1. AYRES, J. C., AND SLOSBERG, H. M. Destruction of Salmonella in egg albumen. *Food Technol.*, **3**, 180 (1949).
2. BROWNE, A. S. The public health significance of Salmonella on poultry and poultry products. (Ph.D. thesis, University of California, Berkeley, California, 1949).
3. CATHCART, W. H., MERZ, A., AND RYBERG, R. E. Staphylococci and Salmonella control in foods. IV. Effect of cooking bakery custards. *Food Research*, **7**, 100 (1942).
4. FELSENFELD, O., YOUNG, V. M., AND YOSHIMURA, T. A survey of Salmonella organisms in market meat, eggs and milk. *J. Am. Vet. Med. Assoc.*, **116**, 17 (1950).
5. GIBBONS, N. E., FULTON, C. O., AND REID, M. Dried whole egg powder. XXI. Pasteurization of liquid egg and its effect on quality of the powder. *Canad. J. Res. F.*, **24**, 327 (1946).

6. GIBBONS, N. E., AND MOORE, R. F. Dried whole egg powder. XXII. The effect of drying, storage and cooking on the Salmonella content. *Canad. J. Res. F.*, **22**, 58 (1944).
7. HINSHAW, W. R., AND MCNEIL, E. Salmonella infection as a food industry problem. *Advances in Food Research.* Academic Press, Inc. New York **3**, 209 (1951).
8. HUSSEMANN, D. L., AND WALLACE, M. A. Studies on the transmission of Salmonella by cooked fowl. *Food Research*, **16**, 89 (1951).
9. LEIFSON, E. New selenite enrichment medium for the isolation of typhoid and paratyphoid (Salmonella) bacilli. *Am. J. Hyg.*, **24**, 423 (1936).
10. SCHNEIDER, M. D. Investigation of Salmonella content of powdered whole egg with not more than two percent moisture content. II. General survey on occurrence of species of Salmonella in high quality egg powder. *Food Research*, **11**, 313 (1946).
11. SCHNEIDER, M. D., AND GUNDERSON, M. F. Investigators shed more light on Salmonella problem. *U. S. Egg and Poultry Mag.*, **55**, 10 (1949).
12. SOLOWEY, M., AND CALESNICK, E. J. Survival of Salmonella in reconstituted egg powder subjected to holding and scrambling. *Food Research*, **13**, 216 (1948).
13. SOLOWEY, M., SUTTON, R., AND CALESNICK, E. Heat resistance of Salmonella organisms isolated from spray-dried whole egg powder. *Food Technol.*, **2**, 9 (1948).
14. WINTER, A. R., STEWART, C. F., MCFARLANE, V. H., AND SOLOWEY, M. Pasteurization of liquid egg products. III. Destruction of Salmonella in liquid whole eggs. *Am. J. Publ. Health*, **36**, 451 (1946).

RESISTANCE OF BACTERIAL SPORES TO GAMMA IRRADIATION

BRUCE H. MORGAN* AND JAMES M. REED
National Canners Association Research Laboratories, Washington, D. C.

(Manuscript received June 9, 1953)

The preservation of foods in their natural state for extended lengths of time after harvest has been a continuing goal. This has meant control of the numerous variables involved such as color, texture, taste, odor, and nutrient values. The basic tools for the preservation of foods have been few; reliance has been placed, for the most part, upon salt, sugar or water concentration, or changes in either pH or temperature. Variations in the application of these principles are continually being made in an effort to produce preserved products that retain the appeal of the natural food at the peak of harvest. An example of this within the canning industry has been the increased use of high temperature-short time sterilization methods.

The canning industry has evidenced definite interest in ionizing radiations as a potential means of food preservation. The work of Proctor and associates *(6)* of the Massachusetts Institute of Technology during the last decade, and more recently the work of others has shown not only some of the limitations of this method but has also outlined its industrial potentialities. In the last several years, added impetus has been given to the industrial use of this mode of sterilization by the possible availability of by-products from the government's atomic energy program which might serve as economical sources for these radiations.

In surveying five possible types of radiations—neutrons, protons, alpha particles, beta particles, and gamma or X-rays—which might be used, it appears that properties of the latter two might be adaptable to high speed industrial sterilization. The comparative properties of beta particles and X-rays are available in the literature *(6, 11)* so it will suffice to point out that an X-ray or gamma ray has far greater penetrating power than a cathode ray or beta particle at the same energy level.

The production of X-rays by particle accelerators is relatively inefficient, making it difficult to obtain economically a large enough flux of X-rays to permit sterilization of foods at the production rate desired by industry. Since radioactive wastes may be available in quantities sufficient to accomplish this in the future, gamma rays produced by radioisotopes were used in the present investigation instead of X-rays.

Results reported by numerous workers have shown that the spore forms of fungi and bacteria are more resistant than vegetative forms to beta and gamma radiation, as they are also to heat. Since the only spore-forming organism of interest to the canning industry from a public health standpoint is *Clostridium botulinum* it would seem important to establish the absolute or practical lethality of gamma radiation with respect to *Cl. botulinum*.

*Present address: Qm. Food and Container Institute for the Armed Forces, Chicago, Ill.

With this in mind, the present investigation has centered on the determination of the relative gamma irradiation resistance of the spores of *Cl. botulinum* in relation to other food spoilage organisms and a study of the environmental factors which might influence the sterilization requirements of these spores.

EXPERIMENTAL PROCEDURE

Representative species of the spore-forming bacteria presently of interest in the canning industry, due to their heat resistance or public health significance, were exposed to gamma radiation from Cobalt 60 or Tantalum 182 sources. The spore formers selected represented the putrefactive anaerobes, including *Cl. botulinum*, Types A and B, the thermophilic anaerobes, and the thermophilic aerobes including the acid tolerant facultative type.

The spore crops were prepared in our Washington laboratories using our standardized spore production procedures *(8, 10)* sealed in glass thermal death time tubes, heat shocked, transported at 5°C. to the Brookhaven National Laboratory for exposures, and returned to Washington for microbiological assay. The thermal death time tube was chosen as an irradiation vessel because of the restrictions of space in the gamma source (13 × 1.7 inches) and the desire to have several replicates per variable during each exposure. Individual points plotted in graphs are averages of from 4 to 20 replicates for each experiment. Dosage rates were reported to us by the Brookhaven staff which originally used the ceric reduction and later the ferrous sulfate oxidation method of chemical dosimetry. In all experiments original spore concentrations used were many times those expected in foods in the course of normal preservation procedures. These higher original populations allowed quantitative definition of survivor curves.

A series of experiments was performed early in our program to validate our methods of exposing spores to gamma rays. The first of these involved determining the amount of lethality received by the 30 to 36 tubes used during any one exposure in terms of spores that were able to reproduce after irradiation. It was found that regardless of the location of the thermal death time tube in the source the same number of spores were killed per unit of exposure time.

Since gamma radiation was available at the Brookhaven Laboratory from the disintegration of either Cobalt 60 or Tantalum 182, the effect of gamma ray source upon spore survival was investigated. In the course of two experiments two Cobalt 60 sources were used (193,000 and 187,000 R./hr.) and three Tantalum 182 sources (367,000, 39,000, 27,000 R./hr.). It was found that regardless of the source of gamma rays—that is, the type of disintegrating element—the lethal effect upon the spores was the same. Also, regardless of the rate of irradiation ranging from 27,000 R./hr. to 367,000 R./hr., the lethal effect of a given roentgen dosage upon the spores was the same.

A comparative study was also made on the recovery of irradiated spores a few days after irradiation and after up to 8 weeks of storage at 4°C. Representative recovery counts for F. S. No. 1518 are listed in Table I, and comparable recovery count variations were obtained with the anaerobes. Within the limits of experimental error due to the small number of tubes counted (one to five for each condition) there appears to be no consistent change in the recoverable number of spores during storage for up to 8 weeks at the refrigeration temperature of 4°C.

SPORE IRRADIATION RESISTANCE

In comparing the irradiation resistance of the spores of three clostridial types (two putrefactive anaerobes, one thermophilic anaerobe) and one bacillus, exposed in phosphate buffer solution (pH 7.0), the survivor curves presented in an earlier paper *(5)* and shown in Figure 1 were determined. It is noteworthy that the most heat resistant organisms are F. S. No. 1518, an aerobe, and P. A. No. 3679, an anaerobe, in that order, and the least heat resistant is *Cl. botulinum*. It would appear that there

TABLE 1

Effect of 4°C. storage on recovery of irradiated spores F. S. No. 1518 in 1:10 dilution of pea puree

Irradiation dose (R)	Before storage	4 weeks	6 weeks	8 weeks
0	33,000,000	44,000,000	22,000,000	.....
55,000	14,200,000	22,000,000	16,000,000	14,500,000
110,000	11,500,000	15,900,000	17,000,000	18,000,000
165,000	6,500,000	10,600,000	7,800,000	8,800,000
441,000	1,440,000	1,170,000	1,600,000	2,800,000
882,000	33,100	20,000	30,000	25,000
1,100,000	1,470	750	3,400	3,000
1,330,000	375	305	450	640
1,540,000	78	10	74	38
1,760,000	4	0	0	0

is no direct correlation between spore resistance to heat and to irradiation. For the purposes of process determination the higher irradiation resistance of *Cl. botulinum* observed in this experiment suggests that a minimum safe process for irradiated foods would result in true sterility rather than commercial sterility.

Assuming that an irradiation process would be based upon true sterility the relationship between the original spore population and the re-

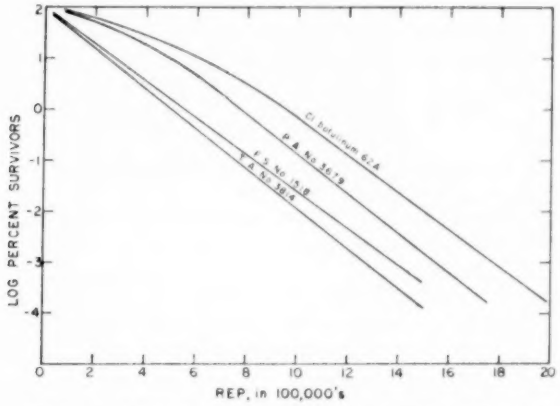


Figure 1. Survival of spores exposed to gamma radiation.

quired irradiation dosage was determined. As previously reported *(5)*, Figure 2 shows the survivor curves for three suspensions of varying initial spore concentration. In confirmation of Fram's *(2)* work, it is noted that the slopes of the three survivor curves are the same, indicating that a given irradiation dose reduces the original population by the same percentage. As in heat sterilization, where the original microbial population and the desired amount of population decrease determine the amount of heat required, so in irradiation the level of the original population will determine the amount of irradiation needed to achieve sterility.

Conditions of Spore Preparation

Several workers *(4, 9)* have reported that the physical and chemical conditions existing during irradiation of microbial forms have affected the sensitivity of these forms of life to irradiation. Hollaender *(3)* has also reported the protective effect upon non-spore forming bacteria of anaerobic growth conditions during cell production prior to irradiation. If these protective effects are of a significant magnitude for spores, they would naturally affect the requirements for sterilization by irradiation.

We have chosen several conditions, both prior to and during irradiation, and used them as variables in following spore sensitivity to gamma radiation.

Using the acid tolerant flat sour organism, *B. coagulans*, which is preferably a mesophilic aerobe, as a test organism the following spore crops

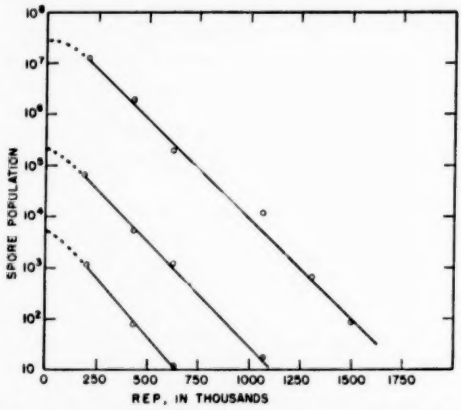


Figure 2. Survival of spores of Flat Sour No. 1518 exposed to gamma rays, using three levels of original population.

were prepared. Two spore crops each were grown at temperatures of 28°C., 37°C., and 55°C. One of these crops at each temperature was grown on an agar medium surface and the other in a liquid medium of similar composition which was mechanically agitated to give maximum aeration during growth. These six spore crops were then irradiated under identical conditions.

The percent survival figures for the spore crops produced at the three temperatures and irradiated in phosphate buffer solution are shown in Table 2. The results indicate that the irradiation resistance of a spore can be affected by the temperature at which the spores are produced and that the lowest growth temperature used for this organism gave spores of the greatest irradiation resistance.

When analyzing the data to determine difference in irradiation sensitivity between spores produced on an agar surface and in aerated medium, it was found, as shown in Table 3, that the spores produced in the aerated liquid medium were more sensitive to gamma rays. This increased sensi-

TABLE 2

Effect of temperature during spore production of B. coagulans upon survival during irradiation

(Percentages are based on mean count of 24 replicates)

Dose (R)	Spores produced at (1) 28°C.	Spores produced at (2) 37°C.		Spores produced at (3) 55°C.	
	% Survival	% Survival	$\frac{(2)}{(1)}$	% Survival	$\frac{(3)}{(1)}$
200,000	5.0	2.90	.58	2.76	.55
500,000	0.163	0.037	.23	0.029	.18
800,000	0.0053	0.00152	.29	0.00147	.28

TABLE 3

Effect of aeration during spore production of B. coagulans upon survival during irradiation

(Percentages are based on mean count of 36 replicates)

Dose (R)	Spores grown on (1) solid agar surface	Spores grown on (2) shaken liquid media	$\frac{(2)}{(1)} \times 100$
200,000	4.0% survival	3.1% survival	77
500,000	0.105% survival	0.048% survival	46
800,000	0.00664% survival	0.00125% survival	19

tivity of spores produced under conditions of maximum aeration might well be due to the amount of oxygen remaining in or on the spore wall.

When the six spore crops were suspended and irradiated in buffer solutions at pH levels of 5, 7 and 8 no consistant differences were noted in the numbers of spores recovered after irradiation.

Conditions of Spore Irradiation

As oxygen present during irradiation has been shown to be toxic to vegetative forms of bacteria, a study of the effect of oxygen on spores was undertaken. Spores of an aerobe (F. S. No. 1518) and an anaerobe (*Cl. botulinum* No. 62-A) were irradiated in thermal death time tubes. The spores were suspended in either phosphate buffer solution or in a solution of lowered oxygen tension consisting of 0.5% aqueous solution of both ascorbic acid and thiourea. The gaseous atmosphere above the liquid surface in the exposure tubes was varied by sealing at atmospheric pressure, sealing under a vacuum of $28\frac{1}{2}$ inches, or evacuating, flushing with nitrogen and sealing in an atmosphere of nitrogen. Thus, the oxygen concentration was varied both in the liquid and gas phase of the tubes during exposure to gamma rays.

In Figures 3 and 4, the logarithm of percent of spores surviving in ascorbic-thiourea solution and in phosphate buffer is plotted against increasing irradiation. These Figures demonstrate the protection offered bacterial spores by irradiating them under reduced conditions. Another point of interest is that the magnitude of the protective effect afforded by the ascorbic-acid-thiourea mixture is somewhat greater for the aerobic than for the anaerobic spores.

Figures 5 and 6 present results from the same experiment. In these Figures, the log percent of spores surviving in tubes sealed under vacuum,

under nitrogen or in air is plotted against increasing irradiation. The gaseous atmosphere in the sealed tube is the significant variable. The lowered oxygen concentrations afforded protection against gamma radiation, with the magnitude of the protective effect being somewhat greater for the anaerobic than for the aerobic spores.

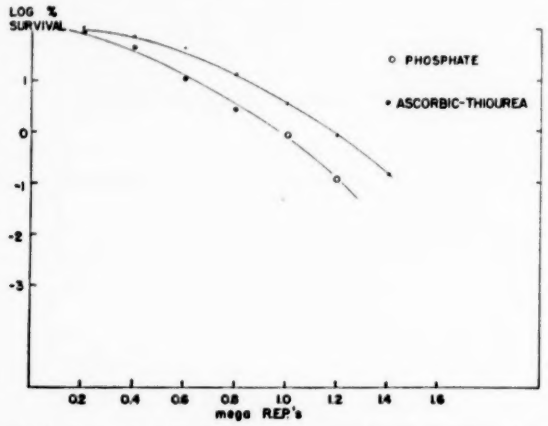


Figure 3. Survival of spores of Cl. botulinum in menstruum of different redox potential.

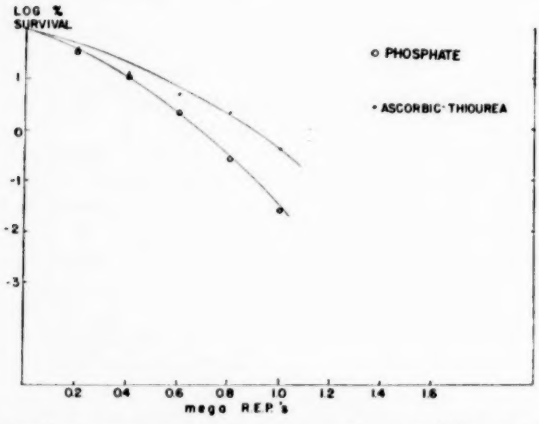


Figure 4. Survival of spores of F. S. 1518 in menstruum of different redox potential.

It then appears that spores of the aerobe and anaerobe under test were protected to some extent from gamma rays by decreasing the concentration of the oxygen in either the liquid substrate or the gas above the substrate. The spores of the anaerobic organism were protected to a greater degree than the aerobe by physically removing gas from the atmosphere above the substrate. The anaerobe was afforded less protec-

tion than the aerobe when the oxygen tension of the suspending substrate was lowered by chemical means.

The results of these experiments involving oxygen seem to indicate that the action of oxygen toxicity might be at the surface or in the cell wall of the spore during irradiation. This would follow from our three major results. First, when the spore is produced under conditions of maximum aeration there is a greater sensitivity to gamma rays. Second, when the oxygen tension in the atmosphere above the surface of a liquid in which spores are suspended is lowered by vacuum or replacement with

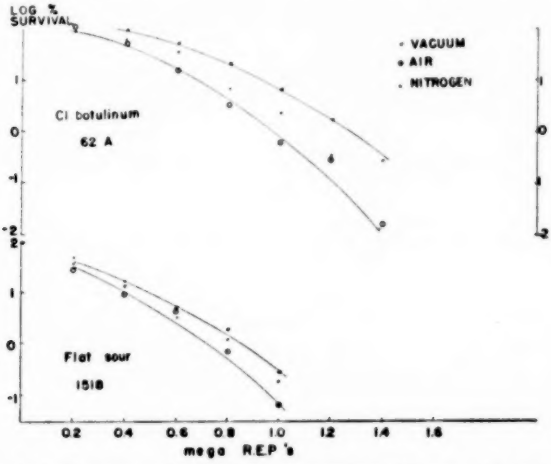


Figure 5. Survival of spores under different gaseous environments (Cl. botulinum 62 A).
Figure 6. Survival of spores under different gaseous environments (flat sour 1518).

nitrogen, more protection is afforded to the anaerobe. If we assume an excess of oxygen around or in the aerobic spores and a minimum of oxygen around or in the anaerobic spores, prior to changing the gaseous atmosphere, it is plausible that a greater proportion of the oxygen would be removed from around the anaerobe. This could still leave a concentration of oxygen around the aerobe that would be toxic. Third, when the oxygen tension in the suspending fluid is chemically lowered[b] more protection is afforded to the aerobe. In this instance the final concentration of oxygen in the proximity of the spore wall of both the aerobe and anaerobe would be approximately equal. This would afford a greater protection to the aerobe, which would have undergone a greater total change in oxygen than the anaerobe. Regardless of the actual mechanism of the protective action afforded by lowered oxygen levels, it would appear that this factor should be considered in connection with the determination of sterilization requirements.

[b] It should be noted that the ascorbic-thiourea solution had the ability not only to reduce the redox potential, but contained substances that have been shown to be protective to enzymes *(1)* or act as "free radical acceptors" *(7)*.

HEAT-IRRADIATION PROCESS

The possibility of using a short heat treatment, either prior to or after irradiation, has been suggested as a means of inactivating the more irradiation-resistant food enzymes.

Using the thermophilic anaerobe No. 3814, the putrefactive anaerobe No. 3679, and the acid tolerant flat sour organism No. 2253, spore suspensions were either exposed to three different levels of irradiation or held as non-irradiated controls. The irradiated and control samples were then heated at 240°F. or 212°F. and the survivor curves plotted. Figure 7

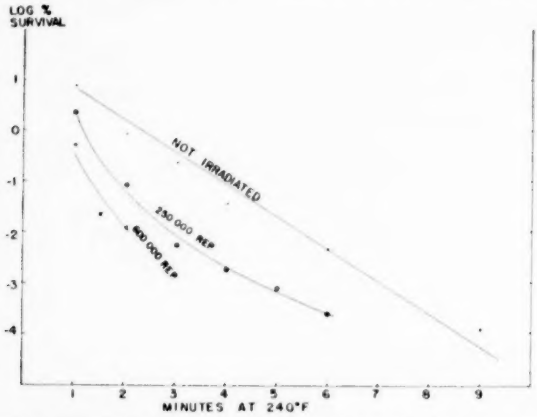


Figure 7. Survival of spores of T. A. 3814 heated after irradiation.

shows the results obtained with T. A. No. 3814 which are typical of the results with the other two organisms. As preliminary irradiation exposure increased, the ability to survive post-irradiation heating decreased. The surviving spores after an irradiation dose of one million roentgens were so few in number that these results are not plotted, but the trend was the same.

The reverse procedure to that just described was then investigated. Spore suspensions of a thermophilic aerobe (Flat sour No. 1518), an acid-tolerant flat sour organism (No. 2253), a thermophilic anaerobe (No. 3814), and a putrefactive anaerobe (No. 3679) were heated to destroy about 80% of the population. Heated and control tubes were then subjected to increasing roentgen dosages. Results plotted as survivor curves in Figure 8 show relatively insignificant differences between the irradiation survivor curves of heated and non-heated spore suspensions for Flat Sour No. 1518 and T. A. No. 3814. These curves are typical of those obtained for all organisms treated.

Results of heat treatment tests in combination with irradiation indicate that heating after irradiation might be more effective than heating before irradiation from the standpoint of total sterilization effects. The heat treatments used in our experiments were more than sufficient for enzyme inactivation, and in view of the results noted further studies along these lines might be worthwhile.

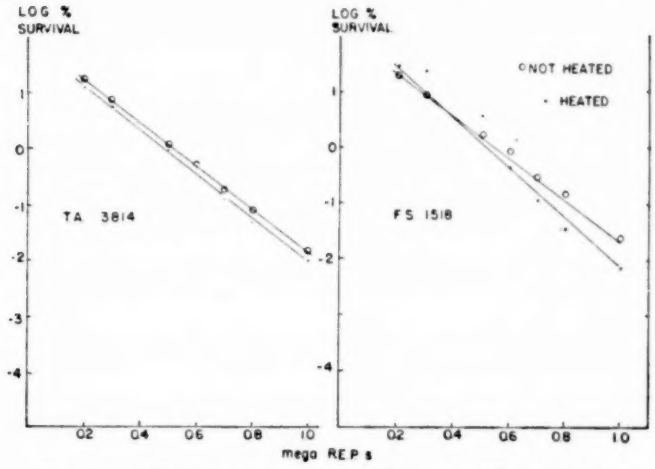


Figure 8. Effect of pre-irradiation heating upon spore survival (T. A. 3814).

Figure 9. Effect of pre-irradiation heating upon spore survival (F. S. 1518).

SUMMARY

The results reported emphasize the importance in gamma ray sterilization studies of thoroughly investigating the effect of conditions during spore production and spore irradiation. Under arbitrarily standardized conditions spores of *Cl. botulinum* were more resistant to gamma radiation than spores of the other food spoilage organisms investigated. However, it was shown that different conditions during spore production or irradiation can increase or decrease the sterilization requirement for a particular type of organism.

In view of these results definition of a sterilizing gamma radiation dosage with respect to spore-forming organisms should assume an environment affording maximum protection.

Acknowledgment

Special acknowledgment is made for the generous assistance given by Dr. J. Yesair and Mr. C. B. Denny of the N.C.A. Research Laboratories in the microbiological assay of irradiated materials.

LITERATURE CITED

1. Dale, W. M. The indirect action of ionizing radiations on aqueous solutions and its dependence on the chemical structure of the substrate. "Symposium on Radiation Microbiology and Biochemistry." *J. Cellular Comp. Physiol.*, **39**, (Supl. 1) 39 (1952).
2. Fram, H., Proctor, B. E., and Dunn, C. G. Effects of X-rays produced at 50 KV on different species of bacteria. *J. Bact.*, **60**, (3), 263 (1950).
3. Hollaender, A., Stapleton, G. E., and Martin, F. L. X-ray sensitivity of *E. coli* as modified by oxygen tension. *Nature*, **167**, 103 (January 20, 1951).
4. Moos, W. S. Variation of irradiation effects on microorganisms in relation to physical changes of their environment. *J. Bact.*, **63**, (5), 688 (1952).

5. Morgan, B. H., and Bohrer, C. W. Sterilization by atomic radiation. National Canners Association, *Information Letter* (No. 1426) (February 28, 1953); *Canning Trade*, **75**, 34 (March 9, 1953).
6. Proctor, B. E., and Goldblith, S. A. Electromagnetic radiation fundamentals and their application in food technology. *Advances in Food Research*, **3**, 120 (1951).
7. Proctor, B. E., Goldblith, S. A., Bates, C. J., and Hammerle, O. A. Biochemical prevention of flavor and chemical changes in foods and tissues sterilized by ionizing radiations. *Food Technol.*, **7**, 237 (1952).
8. Reed, J. M., Bohrer, C. W., and Cameron, E. J. Spore destruction rate studies on organisms of significance in the processing of canned foods. *Food Research*, **16**, (5) (1951).
9. Stapleton, G. E., Billen, D., and Hollaender, A. The role of enzymatic oxygen removal in chemical protection against X-ray inactivation of bacteria. *J. Bact.*, **63**, (6), 805 (1952).
10. Stern, R. M., Hegarty, C. P., and Williams, O. B. Detection of *Bacillus thermoacidurans* (Berry) in tomato juice, and successful cultivation of the organism in the laboratory. *Food Research*, **7**, (3) (1942).
11. Urbain, W. M. Facts about cold sterilization. *Food Eng.*, **25**, (2) (3) (1953).

DETERMINATION OF THE SOLUBILITY INDEX OF SPRAY DRIED EGGS[a]

S. J. BISHOV AND J. H. MITCHELL, JR.
QM Food and Container Institute for the Armed Forces, Chicago, Illinois

(Manuscript received July 15, 1953)

The solubility index of egg powder has been widely used as an index of quality in connection with other chemical and sensory data *(1, 2, 3, 5, 6)*. Most methods for the determination of the solubility index in use at present are empirical in nature. The solubility index described in this paper is a measure of protein material in colloidal or true solution in 0.9% sodium chloride solution. The basis of choice of any method is its reproducibility, rapidity, simplicity, and correlation with functional properties and consumer acceptability. In storage stability investigations of spray dried eggs at the Quartermaster Food and Container Institute, solubility studies by Method I *(4)* were included among such other tests as custard consistency, flavor, sponge cake volume, and emulsification power. Due to difficulties encountered in obtaining reproducible solubility index data, work was initiated to develop a new method. This resulted in a technique which gave more reproducible results and which is, moreover, simple and rapid. This method has been successfully used at the Quartermaster Food and Container Institute Food Research Laboratories in Chicago since 1947.

The modified method, which is modeled after Method II *(4)*, avoids tedious filtration and sedimentation, using centrifugation instead. Precipitation of soluble and colloidal proteins is easily effected by the Esbach reagent used in Method II instead of the buffer reagent as required by Method I. One additional advantage, particularly when large numbers of spray dried egg samples are routinely analyzed, is that the time necessary for the new technique is approximately one-half that required for Method I *(4)*. Since the method under discussion was developed at the Quartermaster Food and Container Institute, Food Laboratories, it will hereafter be referred to in this paper as the QM Method.

PART I

METHOD AND EXPERIMENTAL PROCEDURES

Principle: Egg solids, chiefly proteins of limited particle size, colloidal or in true solution, are precipitated with Esbach reagent and measured volumetrically.

Reagents:

1. Esbach Reagent: 20 g. citric acid and 10 g. picric acid in one liter of water. Picric acid is added to hot water and the flask kept in hot water bath until the picric acid completely dissolves. Citric acid is then added and the solution is made up to one-liter volume at room temperature.
2. 0.9% sodium chloride in distilled water.

[a] This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 438 in the series of papers approved for publication. The views or conclusions contained in this paper are those of the authors; they are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

Experimental Equipment:

1. 150-ml. bottles, glass stoppered.
2. 40-ml. thick-walled centrifuge tubes.
3. 15-ml. graduated centrifuge tubes.
4. Shaking apparatus, 240 cycles/minute, 4-inch amplitude.
5. Centrifuge, size No. 1 International, 16" head.

Minimal Alternate Experimental Equipment:

1. 250-ml. bottles or flasks, rubber stoppered.
2. 15-ml. graduated centrifuge tubes.
3. Centrifuge other than No. 1 International.

Calibration of Solubility Index Curve of Stabilized* and Non-Stabilized Egg Powder: Samples of zero solubility spray dried eggs were prepared by heating spray dried egg powder in a drying oven at 110°C. for 16 hours. These and freshly spray dried powder of good quality were used to obtain data of Table 1 in the experimental procedure following.

The proportions of the denatured and the non-denatured powders per 100 ml. 0.9% saline used, the relation to solubility index, and percent solubility are shown in Table 1 and in Figures 1 and 2.

TABLE 1

Relationship of solubility index to per cent solubility

Composition of powders of decreasing solubility		Solubility index		Percent[c] solubility
Heat denatured powder	Fresh powder	Stabilized[b]	Non-stabilized[b]	
g.	*g.*			
0	3.0	1.60	1.52	100
0.6	2.4	1.32	1.23	80
1.2	1.8	.99	.97	60
1.8	1.2	.67	.69	40
2.4	0.6	.33	.39	20
3.0	0	0	0	0

[b] Mean value.
[c] Calculated.

EXPERIMENTAL PROCEDURE

The procedure, as developed, is given below:

An accurately weighed 1.50-g. sample of egg powder is placed in a 150-ml. glass-stoppered bottle containing 50 ml. 0.90% sodium chloride solution. Where no 150-ml. bottles are available, any suitable piece of laboratory glassware may be used which will permit adequate stirring for complete dispersion.

The bottles and contents are agitated for 2 minutes, preferably in a shaking machine (250 cycles per minute). After standing for 5 minutes, the shaking is repeated for an additional 2 minutes. If a shaking machine is not available, the bottles should be slowly inverted 75 times by hand, allowed to stand 5 to 10 minutes, and again slowly inverted 75 times. (Improper shaking may become an important source of error. Shaking 50 times by inverting the bottles should suffice, but must be continued for as many times beyond 50 as are required to break any gross egg-particle aggregates.) The samples are immediately poured into a 40-ml. thick-walled calibrated centrifuge tube up to the 40-ml. mark, and centrifuged in a No. 1 International centrifuge, having a head 16 inches in diameter, at 2,000 r.p.m. for 10 minutes. The necessary r.p.m. should be attained within 30 seconds or some fixed time interval, preferably not much over 30 seconds. Five ml. of the supernatant liquid are transferred to a 15-ml. graduated centri-

*Stabilized egg powders are prepared by treatment of the egg with hydrochloric acid before spray drying and subsequent addition of sodium bicarbonate to the dry egg powder in accordance with Quartermaster Corps Specification MIL-E-10006(QM), 3.5.3-3.5.4.

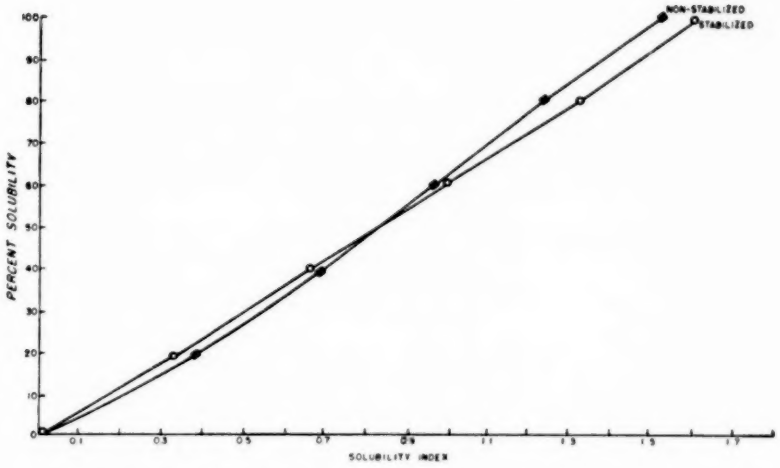


Figure 1. Relations of solubility index and percent solubility to egg powder quality.

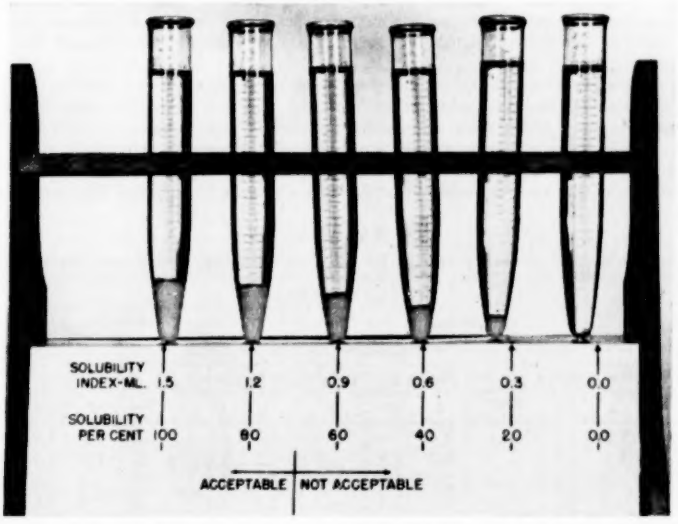


Figure 2. Illustrating percent solubility of stabilized and non-stabilized egg powder.

fuge tube, and 10 ml. of Esbach reagent added and mixed by stirring briefly with a slender glass rod. (Enough supernatant material is available for check determinations.) The tubes are then centrifuged for 10 minutes at 2,000 r.p.m. The centrifuge should be allowed to stop without braking. Sudden braking may disrupt the surface of the centrifugate, making accurate reading of precipitate volume difficult. The volume is read to the nearest one-tenth ml., and recorded as the solubility index.

If a centrifuge of different head diameter is available, the proper speed is calculated as follows:

$$y = 2{,}000\sqrt{\frac{16}{x}}$$

y = r.p.m. desired
x = diameter of head in inches

The "diameter of head" is the distance between the inside bottoms of opposite cups measured through the center of rotation of centrifuge head while the cups are horizontally extended. Variations in centrifuge and centrifuge techniques are the primary source of error and inconsistency of results. Any centrifuge except that with a conical head may be employed provided the R.C.F. (Relative Centrifugal Force) is maintained at 902 g. cm/t^2.

$$R.C.F. = 1.11 \times 10^{-6} Nr$$

N = r.p.m.
r = radius of axis to extremity of tube, in centimeters

PART II

COLLABORATIVE TEST OF METHOD

A collaborative test of the QM Method I *(4)* was conducted in cooperation with the Dairy Branch Laboratory of the United States Department of Agriculture in Chicago (Table 2).

Representative data from 8 manufacturers' samples of spray dried whole eggs, picked at random from an average day's run are presented in Table 2. All values of solubility index were read to the nearest 0.1 ml. on the calibrated 15-ml. centrifuge tubes.

Examination of the data in Table 2 indicates closer aliquot reproducibility of solubility index constants by the QM Method than by Method I *(4)*. The range of variation and the probability of reproducible duplicate determinations indicate that the QM Method is superior to Method I. An analysis of variance corroborated the statistical results given in Table 2.

In a 2-year storage study of stabilized and non-stabilized egg powder Method I gave values below the acceptable range, both in this and other laboratories, for the stabilized products which were manifestly acceptable, and indeed, superior in quality to non-stabilized egg similarly stored. Method I was, therefore, abandoned for the QM method in our later work. The data in Table 3 are given to illustrate the relative applicability of the two methods under discussion to stored stabilized egg powders.

TABLE 2

Comparison of solubility indices by the Quartermaster and Stuart Method I

Sample number	Solubility Index											
	QM Method						Method I					
	F&CI[a]			USDA[b]			F&CI			USDA		
	Aliquot		Variation[f]	Aliquot		Variation	Aliquot		Variation	Aliquot		Variation
	I	II		I	II		I	II		I	II	
1	1.3	1.4	−.1	1.6	1.6	0	1.5	2.5	−1.0	1.3	1.5	−.2
2	1.4	1.5	−.1	1.5	1.5	0	1.7	1.6	+ .1	1.6	1.3	+.3
3	1.5	1.4	+.1	1.6	1.6	0	1.5	1.5	0	1.4	1.2	+.2
4	1.4	1.5	−.1	1.7	1.6	+.1	1.5	2.3	− .8	2.0	2.5	−.5
5	1.7	1.6	+.1	1.7	1.4	+.3	1.8	2.1	− .3	1.9	2.3	−.4
6	1.5	1.4	+.1	1.8	1.7	+.1	1.8	1.7	+ .1	1.3	1.5	−.2
7	1.5	1.6	−.1	1.6	1.7	−.1	2.1	1.8	+ .3	2.0	2.2	−.2
8	1.6	1.5	+.1	1.8	1.6	+.2	2.1	2.3	− .2	2.0	1.8	+.2
Range of variation.....	−0.1 to +0.1			−0.1 to +0.2			−1.0 to +0.3			−0.5 to +0.3		
Std. dev.....	±0.1			±0.1			±0.4			±0.3		

[a] Food and Container Institute Laboratories.
[b] United States Department of Agriculture Laboratories.
[f] Variation of reproducibility of successive aliquots.

Table 4 illustrates the applicability of the QM method to powders of a wide range of solubility. Examination of the data in this table indicates that poor reproducibility is obtained more frequently with powders of high solubility index when Method I is applied than when the QM Method is used.

Table 5 illustrates the relation of solubility index and flavor score of custard made of spray dried whole egg powders which had been stored at 100°F. for 16 months. Both stabilized and non-stabilized powders were tested.

The solubility index data in Table 6 show that the changes occurring in egg powder stored at 100°F. are reflected in the lowered solubility indices after 6 months' storage.

TABLE 3

Comparison of Stuart Method I and Quartermaster Method on stabilized dried egg samples stored two months

Storage temperature, (°F.)	Solubility Index[g]		Palatability Score		
	Stuart Method	QM Method	Scrambled eggs	Sponge cake	Custard
40	.80	1.40	7[h]	7.1[i]	8.7[j]
70	.80	1.40	7	6.5	8.8
100	.82	1.30	6	6.1	7.8

[g] Egg powder with solubility index of over 1.2 is considered good; under 1.0 is considered poor.
[h] Fresh scrambled egg gave score of 7.5.
[i] Fresh egg sponge cake gave score of 8.0.
[j] Fresh egg custard gave score of 8.1.

TABLE 4

Solubility index values and palatability scores of egg powders of wide range of solubility

Sample number	Palatability score[k]	Solubility Index			
		Stuart Method I		QM Method	
		a	b	a	b
1	7.0	1.6	2.0	1.5	1.6
2	7.0	1.4	1.9	1.5	1.5
3	6.5	1.7	1.9	1.6	1.7
4	6.5	1.2	1.2	1.4	1.4
5	6.0	0.9	1.0	0.9	1.0
6	6.0	1.1	1.1	1.2	1.2
7	6.0	1.0	1.0	1.0	1.1
8	5.0[l]	1.0	1.1	1.0	1.0
9	6.0	1.0	1.0	1.1	1.2
10	6.0	1.0	1.1	1.0	1.1

[k] Palatability score of scrambled egg on basis of eight as the highest score. Five was not acceptable; six and up acceptable.
[l] Solubility index of 1.0 and up acceptable but when P. S. less than six not acceptable.
[m] The above samples 1 through 4 were fresh manufacturers' samples, while samples 5 through 10 were storage samples.
NOTE: All samples were obtained from U. S. Department of Agriculture Dairy Laboratory in Chicago.

TABLE 5

Effect of storage on solubility index and custard score, Quartermaster Method

Months of storage at 100°F.	Non-Stabilized		Stabilized	
	Solubility index	Custard[n] score	Solubility index	Custard[n] score
0	1.6	8.0	1.6	8.0
4	1.6	7.1	1.5	7.0
8	1.2	7.5	1.4	8.0
12	0.9	5.8	1.3	6.4
16	0.9	5.6	1.1	6.6

[n] Custard score was based on score of ten for those made with fresh whole eggs

At 40 and 70°F. these changes are not shown until after one year of storage. It may then be concluded that this method of determining solubility index should be useful in evaluating storage stability of egg powders when used in conjunction with other physical, chemical, and taste tests.

TABLE 6

Solubility index constants of stabilized egg powder in storage

Sample	I[a]			II[b]			III[b]		
Months of storage	0	6	12	0	6	12	0	6	12
Temperature °F.	Solubility Index Quartermaster Method								
40°	1.60	1.60	1.40	1.50	1.65	1.30	1.60	1.55	1.30
70°	1.60	1.50	1.40	1.50	1.45	1.30	1.60	1.45	1.30
100°	1.60	1.00	.60	1.50	.....	1.20	1.60	1.35	1.00

[a] 4% moisture eggs.
[b] 2% moisture eggs.

SUMMARY AND CONCLUSIONS

A rapid, reproducible method for determining the solubility of spray dried whole egg powder has been described. The method requires, in addition to an accurate tachometer and centrifuge, only routine laboratory equipment and easily obtainable reagents. The method involves 4 main steps:

a. Dispersion of egg solids in 0.9% saline solution.
b. Centrifugation of saline-insoluble solids.
c. Precipitation of saline-soluble and colloidal fraction with Esbach Reagent.
d. Centrifugation of Esbach-precipitated solids and reading the volume of the precipitate in milliliters to the nearest tenth, as solubility index.

Stabilized and non-stabilized egg powders lend themselves equally well to solubility index determination by this method. Replicate determinations can be readily performed on single weighings of sample. Solubility index values of over 1.5-1.6 corresponding to more than 100% solubility are rarely encountered.

Acknowledgment

Grateful acknowledgment is made to Dr. T. I. Hedrick and Mrs. Edith L. Litt, of the Dairy Branch Laboratory, U. S. Department of Agriculture, Chicago, for technical assistance, in these collaborative studies.

LITERATURE CITED

1. Conrad, P. M. *et al.* Improved Dried Egg Products. *Technical Bull. No. 64*, Agricultural Experiment Station, Kansas State College of Agriculture and Applied Science, Manhattan, Kansas, pp. 25-26 (March, 1946).
2. Hawthorne, J. R. A refractometric method for solubility index. *J. Soc. Chem. Ind.*, **63**, 6 (1944).
3. Stuart, L. S., Goresline, H. E., Smart, H. E., and Dawson, V. T. Relation of egg powder quality to change in solubility. *Food Inds.*, **17**, 154-6, 242 (1945).
4. Stuart, L. S., Grew, E., and Dicks, E. E. Solubility of spray dried whole egg powder. *U. S. Egg Poultry J.*, **48**, 498 (1942).
5. Thistle, M. W., Pearce, J. A., and Gibbons, N. E. Dried whole egg: Method of assessing quality. *Can. J. Research*, **D-21**, 1-7 (1943).
6. White, G. A. A refractometric method for the determination of solubility. *Can. J. Research*, **D-21**, 203 (1943).

ANTIOXIDANT ACTIVITY AND ANTAGONISM TO MICROBIAL GROWTH BY PIPERINE, ISONICOTINYL HYDRAZIDE AND RELATED COMPOUNDS

TORSTEN HASSELSTROM, G. P. DATEO, H. S. LEVINSON AND W. H. STAHL

Pioneering Research Laboratories, U. S. Army Quartermaster Corps, Philadelphia, Pennsylvania

AND

E. J. HEWITT AND K. S. KONIGSBACHER

Evans Research and Development Corporation, New York 17, N. Y.

(Manuscript received November 18, 1953)

Evaluation of the effectiveness of spices in preventing food spoilage has primarily dealt with the activity of the whole spice, rather than with its components and related chemicals. Dubois and Tressler *(3)* found that black pepper retarded rancidity in frozen ground pork and beef. Chipault *et al. (1)* observed that black pepper has an antioxidant effect on lard, but that the petroleum-ether and the alcohol soluble portions did not exhibit activities as marked as the ground spice.

In the present investigation, the antioxidant activity and antagonism to fungal and bacterial growth of ground black pepper spice, components of the spice and some chemicals related to piperine, the natural peppery-bite compound of *Piper nigrum*, were explored. The black pepper oleoresin was obtained from the ground spice by ether extraction and subsequent removal of the oil portion from the ether extract. Apopiperine or β-cinnamenylacryloyl piperidide was prepared according to Staudinger and Schneider *(8)*; β-cinnamenylacryloyl hydrazide, isonicotinyl hydrazide and 5-phenyl-3-pentenoyl hydrazide were also included in the series. The three latter compounds were chosen because of their structural relationship to piperine; isonicotinyl hydrazide has, in addition, a slight peppery bite. To aid in locating the origin of the antioxidant activity in black pepper spice, the oleoresin was catalytically hydrogenated and acetylated and the products tested for their behavior as antioxidants.

EXPERIMENTAL

Antioxidant activity. Values given in Table 1 on freshly distilled pepper oil (residue from ether extract of steam-distillables from freshly ground pepper*) were those obtained after allowing the samples to stand at room temperature for 48 hours.

The oleoresin was secured by ether extraction of the non-steam volatile residue, drying the ether solution with anhydrous sodium sulfate, and distilling off the solvent. It may be noted that the ratio of pepper oil to oleoresin naturally occurring in black pepper spice is approximately 2 to 5.

Table 2 describes the results on prime distilled lard. Measurements of antioxidant activity were made by Riemenschneider's modification *(7)* of the Swift Stability Test. Samples were removed at suitable intervals and peroxide determined in all tests by the method of Wheeler *(10)*.

*Lampong variety of black pepper spice. The sample was secured from McCormick & Company, Inc., Baltimore, Maryland.

TABLE 1

Antioxidant activity of pepper oleoresin on pepper oil

Sample	Oil (g.)	Oleoresin (g.)	Atmosphere	Peroxide, meq. per 1,000 g. oil
1	5	None	N_2	None
2	5	None	O_2	160
3	5	5	O_2	13
4	5	10	O_2	7

The tocopherol content of black pepper oleoresin. Using the Wall and Kelly modification *(9)* of the Emmerie and Engel procedure, it was shown that the black pepper oleoresin contained 0.1% α-tocopherol and 0.54% total tocopherol.

Antagonism of microbial growth. Appearance of colonies of *Micrococcus pyogenes* var. *aureus* (QM B1458) on nutrient agar (Difco) after 18 hours in the presence of varying concentrations of the spice and the chemicals was used as the criterion of antibacterial activity. *M. pyogenes* was selected as the test organism in these experiments since the staphylococci are, according to Dack *(2)*, the most common cause of food poisoning; meats have often been implicated in outbreaks of such poisonings.

TABLE 2

Antioxidant values on steam-distilled lard

(Lard control—5.5 hours)

Substance tested	Concentration, per cent		
	0.1	0.01	0.001
	Antioxidant value[2]		
Nordihydroguiaretic acid[1]	.....	.....	72.0
Propylgallate[1]	.....	.....	40.0
Tocopherol[1]	12.0	.....	.....
Pepper	6.5		
Pepper oleoresin	13.5		
Hydrogenated oleoresin	7.5		
Acetylated oleoresin	4.5		
Piperine	5.5		
β-cinnamenyl acryloyl piperidide	5.0		
β-cinnamenyl acrylic acid	4.5		
β-cinnamenyl acryloyl hydrazide	25.0		
5-phenyl-3-pentenoyl hydrazide[3]	18.0		
Isonicotinyl hydrazide	105.0	15.0	5.0

[1] Commercially available antioxidants.

[3] The 5-phenyl-3-pentenoylhydrazide was prepared in the following manner: 1 ml. of methyl ester of 5-phenyl-3-pentenoic acid was added dropwise to 6.0 ml. of hydrazine hydrate (85% solution). To this was added 7.0 ml. of methanol and the solution heated to boiling. The excess solvent was evaporated, leaving a solid which was recrystallized from petroleum ether to constant melting point. M.p. 67.5-68°. Analysis: Calculated for $C_{11}H_{14}ON_2$: N, 14.71. Found: N, 14.77; 14.71.

[2] Hours elapsed until onset of rancidity.

One ml. of a suspension of the test organism containing approximately 4,500 cells was pipetted into duplicate sterile petri plates. The agar-pepper mixture (cooled to 40-45°C.) was poured on the bacterial suspension. The petri plates were incubated at 30°C. for 24 hours, and the colonies in a defined area of the plate were counted.

The amount of growth of *Aspergillus versicolor* (QM 432) in shake flasks containing 3% glycerol in a nutrient salt solution[b] and varying concentrations of the com-

[b] The nutrient salt solution has the following composition NH_4NO_3, 1.00 g.; $MgSO_4 \cdot 7H_2O$, 0.30 g.; KH_2PO_4, 1.77 g.; K_2HPO_4, 15.16 g.; and Bacto yeast extract, 0.10 g. distilled water, q. s. 1 liter

TABLE 3

Bacteriostatic and fungistatic activity

	Against *M. pyogenes* var. *aureus*				Against *Asp. versicolor*		
	Concentration, per cent						
	0.1%	0.01%	0.001%	Control	0.1%	0.01%	Control
	Number of colonies				Relative quantity of growth[1]		
Standard pepper	79	300	322	336	—	++	++++
Pepper oleoresin	0	184	302	336	—	++	++++
Piperine	....[2]	320	322	336	—	+++	++++
β-cinnamenyl-acryloyl-piperide	0	0	238	336	—	+	++++
β-cinnamenyl-acryloyl-hydrazide	40	290	358	336	—	+	++++
Isonicotinyl hydrazide	341	332	328	336	—	+++	++++
Tocopherol	226	287	350	336	—		

[1] ++++ = heavy growth; +++ = medium growth; ++ = thin growth; — = no fungal growth.

[2] Count obscured by the separation of crystalline piperine.

pounds being investigated was used as the criterion of antifungal activity. The flasks were incubated on a shaker at 30°C. for 7 days. Results of the microbial and fungal test series appear in Table 3.

RESULTS AND DISCUSSION

Black pepper spice, pepper oleoresin, piperine, apopiperine, β-cinnamenyl acryloyl hydrazide and isonicotinyl hydrazide allowed no fungal growth at 0.1% concentrations; at 0.01% concentrations the growth was appreciably retarded. Pepper oleoresin and apopiperine are bacteriostatic at the same concentrations. Ground black pepper spice at 0.1% concentrations is antagonistic toward growth of bacteria. As expected, isonicotinyl hydrazide showed no bacteriostatic properties, as it is known that this compound is specifically bacteriostatic to *Mycobacterium tuberculosis (4, 6)*. Pepper oleoresin, β-cinnamenylacryloyl hydrazide, 5-phenyl-3-pentenoyl hydrazide and isonicotinyl hydrazide showed definite antioxidant activity in concentrations of 0.1%. The isonicotinyl hydrazide showed this property to a surprisingly high degree, being approximately ten times higher than that of pepper oleoresin and the tocopherols.[c] Since acetylated and hydrogenated black pepper oleoresin showed no antioxidant activity, it was concluded that the tocopherols of black pepper are responsible for its anti-rancidity property. It appears that black pepper spice in concentrations commonly used in foods does not prevent food deterioration. However, the good storage qualities of the spice—namely, resistance to

[c] The rather high antioxidant activity exhibited by isonicotinyl hydrazide was not expected. *Mycobacterium tuberculosis* is singular in the respect that it produces a waxy outer layer or capsule which contains as high as 85% oxygenated fats *(5)*. The formation of oxygenated fats is prevented by antioxidants. There may exist a relationship between the antioxidant activity of isonicotinyl hydrazide and its bacteriostatic activity against *M. tuberculosis*, by virtue of possibly preventing the formation of these oxygenated fats with subsequent loss of virulence or inhibition of growth of the organism. Streptomycin, which in itself is not specific for *M. tuberculosis* but used synergistically with isonicotinyl hydrazide in tuberculosis therapy, has no antioxidant activity, as was found in this laboratory.

The above comments are mainly heuristic; those interested and more qualified are invited to continue the investigation.—*Torsten Hasselstrom.*

oxidation and microbiological growth—are evidently due to its high concentration of tocopherols and oleoresin.

LITERATURE CITED

1. CHIPAULT, J. R., MIZUNO, G. R., HAWKINS, J. M., AND LUNDBERG, W. O. The antioxidant properties of natural spices. *Food Research*, **17**, 1 (1952).
2. DACK, G. M. *Food Poisoning*, 1943. University of Chicago Press, Chicago, Ill.
3. DUBOIS, C. W., AND TRESSLER, D. K. Seasonings, their effect on maintenance of quality in storage of frozen ground pork and beef. *Proc. Inst. Food Technol.*, 202 (1943).
4. GREENBERG, E., AND SCHNITZER, R. J. Studies on the activity of hydrazine derivatives of isonicotinic acid in the experimental tuberculosis of mice. *Quart. Bull.*, Sea View Hosp., **13**, 3 (1952).
5. KNAYSI, G. *Elements of Bacterial Cytology*. 1951, Comstock Pub. Co., Ithaca, N. Y.
6. PANAY, F., STANDER, H., AND DONOVICK, R. *In vitro* studies on isonicotinic acid hydrazides, *Am. Rev. Tuberc.*, **65**, 761 (1952).
7. RIEMENSCHNEIDER, R. W., TURER, J., AND SPECK, R. M. Modifications of the Swift stability test. *Oil and Soap*, **20**, 169-171 (1943).
8. STAUDINGER, H., AND SCHNEIDER, H. On the relationship between constitution and pepper flavor. *Ber.*, **56**, 699 (1924).
9. WALL, M., AND KELLY, E. G. Determination of tocopherol in plant tissue. *Ind. Eng. Chem., Anal. Ed.*, **18**, 198-201 (1946).
10. WHEELER, D. H. Peroxide formation as a measure of autoxidative deterioration. *Oil and Soap*, **9**, 89-97 (1932).

HEAT RESISTANCE IN LIQUID EGGS OF SOME STRAINS OF THE GENUS *SALMONELLA*[a,b]

A. ANELLIS, J. LUBAS, AND MORTON M. RAYMAN
Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois

(Manuscript received November 21, 1953)

Thermal destruction of the *Salmonella* group of micro-organisms, which are commonly present in commercially broken out raw egg pulp, is the prime objective of pasteurizing liquid egg in the manufacture of frozen and dried egg products. It is clearly recognized from *Salmonella* pathogenicity studies by McCullough and Eisele *(24, 25, 26, 27)* and from other public health investigations that there is a need for eliminating these offending bacteria from egg products in order to prevent the occurrence of salmonellosis in institutional and military feeding and to minimize the latent "carrier state" of infectiousness, particularly among the food handlers. Adequacy of pasteurization depends on many factors, such as numbers and types of *Salmonella* present in the raw material, the pH of the liquid egg, and the time-temperature relationships involved in the heat resistance of these bacteria.

Thermal process evaluations for food sterilization were established on a scientific basis in 1920 following the classical paper by Bigelow, Bohart, Richardson, and Ball *(7)*. The ensuing three decades have brought many improvements in the technique of heat resistance determinations *(1, 8, 14, 34, 37, 43, 48)* and in the mathematical treatments of the thermal death time data secured *(2, 3, 4, 7, 15, 16, 17, 20, 21, 22, 30, 32, 38, 39, 44, 45, 46)*. Although the literature concerning the thermal resistance of bacterial spores is rich in these advances, it is unfortunate that the same principles have not been as widely adopted by bacteriologists studying the non-sporulating organisms. In connection with their investigation of thermal death time curves of coliform bacteria in milk, Olson, Macy, and Halvorson *(29)* stated recently: "With few exceptions the data on the thermal destruction of non-spore-forming bacteria are of such a nature that thermal death-time curves for the organisms studied cannot be constructed."

A thermal death time curve defines the heat resistance of an organism at a given temperature (F value) as well as its sensitivity to changes in temperature (z value). Such a curve, however, assumes the existence of absolute time-temperature relationships for the total destruction of a particular bacterial concentration. Hence, a sufficient change in the initial population of the suspension undergoing heat treatment would produce a change in the properties of the "absolute" thermal death time curve. A

[a] This paper reports research undertaken by the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned No. 379 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

[b] Presented at the Twelfth Annual Meeting of IFT, Grand Rapids, Michigan, June 11, 1952.

recent development in the interpretation of heat resistance data which is based upon the concepts of Ball *(4)*, Katzin, Sandholzer, and Strong *(22)*, Gillespy *(15, 16)*, and Stumbo *(44, 45)* regards death by heat as a strictly logarithmic process. The logarithmic rate of death is characterized by a D value, or the time required to reduce the original bacterial population 90%. D values are independent of the initial concentration of organisms, as is also the heat resistance curve obtained by plotting log D against temperature.

For comparing the degree of tolerance to heat of the *Salmonella* egg suspensions studied, we have adopted 140°F.(60°C.) as the reference temperature. This chosen standard has previously been applied to the pasteurization of beer *(12)*. The heat resistance data will be presented in accordance with both concepts, and will be expressed by the following symbols:

F_{140} = number of minutes required to completely destroy a known bacterial load at 140°F.(60°C.), assuming instantaneous heating of the cell suspension to 140°F. and instantaneous cooling to a non-lethal temperature.

z_F = slope of the thermal death time curve (F curve), represented by the number of degrees Fahrenheit required for the F curve to pass over one log cycle on semilog paper when temperature is plotted on the linear scale and time on the log scale.

D_{140} = number of minutes required to destroy 90% of the initial bacterial load at 140°F., assuming instantaneous heating of the cell suspension to 140°F. and instantaneous cooling to a non-lethal temperature.

z_D = slope of the decimal reduction time curve (D curve), represented by the number of degrees Fahrenheit required for the D curve to pass over one log cycle on semilog paper when temperature is plotted on the linear scale and D values in minutes plotted on the log scale.

The establishment of an efficient thermal process is dependent upon accurate heat resistance data and a known initial concentration of organisms in the food substrate. The accumulation of reliable thermal resistance data should be based upon the following requirements:

(a) Rate of heat penetration data of the food in question over the heating range selected, for the purpose of correcting the exposure time *(40)*.

(b) For each experiment a sufficient number of replicate test samples (preferably more than 5) for heat treatment at each period of exposure over the temperature and time range employed, in order to eliminate false negative results due to "skips," and

(c) Adequate methods of recovering heat-injured but still viable cells (or spores).

Careful adherence to the above stipulations should yield data which will give straight line thermal death time curves on semilog paper.

The literature on the pasteurization of liquid whole egg for the purpose of reducing or entirely eliminating the number of undesirable bacterial contaminants has been reviewed recently by Goresline *et al. (18)* and Winter *(50)*. Almost all of the pasteurization studies were carried out in commercial or laboratory-type vat, plate, or tubular heaters. One of the most extensive of such investigations was made by Winter *et al. (51)*. They determined by means of a tubular heat exchanger the thermal stability of 164 cultures of 16 different *Salmonella* serotypes. No attempt was made to express their results in the mathematical terms of a point and a slope, although certain of the time-temperature relationships could be converted to thermal death time curves of approximate accuracy. Furthermore, as has been pointed out by the authors, they encountered difficulties in maintaining the exact temperature and time relationships in different experimental runs because of variations experienced in the operation of the pasteurizer.

Much of the data resulting from similar investigations are of a nature which does not permit the construction of heat resistance curves. Solowey, Sutton, and Calesnick *(41)*, however, calculated decimal reduction times according to the method of Katzin *et al. (22)* for egg suspensions of 95 strains of *Salmonella* representing 24 serotypes. They secured the information by employing duplicate samples of cotton-stoppered tubes for each heating period, but unfortunately they presented the results in a way which cannot be converted to heat resistance curves. Nor did they appear to make corrections for heat penetration lag.

Investigations have shown that the stability of dehydrated egg can be improved, without impairing its functional properties, by means of acidifying liquid whole egg with HCl down to pH 5.5 prior to spray drying *(9, 11, 33, 35, 42)*. The Quartermaster Corps utilized this knowledge in a specification *(28)* for the purchase of dehydrated eggs. Since practically all of the pasteurization studies were performed on untreated raw egg (pH range 7.5 to 7.9 when commercially fresh), little information was available on the heat resistance of *Salmonella* in the acidified pulp. Winter *(49)* appears to have been the first to report on the heat resistance of three *Salmonella* serotypes in pH 5.5 melange. An examination of his results indicated that the organisms were less susceptible to heat in pH 5.5 egg than in pH 7.2 egg. A recent preliminary report by Osborne, Straka, and Lineweaver *(31)* also suggested that *Salmonella* is more heat stable in substrates in the pH range 5.5 to 7.5 than in the range 8 to 9.

Because of the paucity of information on the sensitivity of *Salmonella* to heat in acid-treated liquid egg, the purpose of this investigation was to collect thermal resistance data on some of these bacteria in acidified melange, and to determine the influence of pH on the susceptibility of the test organisms to pasteurization temperatures.

METHODS AND MATERIALS

Organisms. The *Salmonella* cultures employed in this study were originally isolated from spray-dried eggs.[c] Strains of *S. oranienburg* 1011, *S. montevideo* 1162,

[c]All organisms were serologically confirmed through the courtesy of Mrs. Mildred M. Galton, Florida State Board of Health.

S. worthington 20A2, and *S. typhimurium* 81 were isolated by Miss Anne F. Byrne of this Laboratory. Cultures of *S. meleagridis* 5485 and *S. senftenberg* 523, 775W, 2623, and 3252 were obtained from Dr. H. Lineweaver, Western Utilization Research Branch, Albany, California. *S. pullorum* 13117 was obtained from Major M. D. Schneider, QMC.

Preparation of sterile, liquid whole egg. Twelve fresh shell eggs were individually washed with warm soapy water in order to remove dirt from the shells and placed in a perforated plastic egg tray. The loaded tray was first submerged for 3 minutes in a 1:100 dilution of Clorox (approximately 100 p.p.m. of available chlorine), drained, and transferred to a bath of 95% alcohol. The eggs were picked up singly with a sterile spoon and flamed; each egg was then held at the ends so as to avoid contaminating the remainder of the shell, cracked open over a horizontally fixed sterile knife, edge up, and the contents emptied into a sterile Waring blender jar. The eggs were emulsified for 5 minutes with the stirring speed adjusted with a Powerstat transformer (30-40 volts) to produce a minimum amount of foaming. A quantity of sterile HCl or NaOH, diluted so as to require a volume of 20 to 30 ml. for the adjustment of the pH of the melange (575-600 ml.) within the range 5.5-9.0, was then pipetted aseptically into the blender jar with continuous mixing of the contents for an additional 5 minutes. The egg pulp was filtered aseptically by suction through a sterile Gooch funnel fitted with 4 layers of sterile cheesecloth into a tared, sterile, one-liter Erlenmeyer flask in order to remove the particles of egg membrane which would clog the valve and needle of the automatic pipetting machine. The flask of liquid egg was held for 15 minutes with intermittent agitation in a 140°F.(60°C.) constant temperature glycerol bath, and then cooled in running water. This heat treatment ensured the sterility of the melange. Heat resistance experiments on a *Salmonella* suspension in such heat-treated egg produced results similar to those found in unheated egg. A 40- to 50-g. egg sample was reserved for heat penetration measurements; one portion (10 ml.) was also pipetted aseptically for checking the final pH and a second sample (5 ml.) for determining the sterility of the product in tryptose broth (Bacto-tryptose 2.0%, sodium chloride 0.5%, glucose 0.2%, and thiamin hydrochloride 0.05%). The melange remaining was weighed and refrigerated until needed. The egg having a pH of less than 7.0 was used within 1-3 days, and the egg with a pH greater than 7.0 was used within one day of preparation. It has been observed that the egg with a pH above 7.0 darkens considerably after 24 hours in the refrigerator.

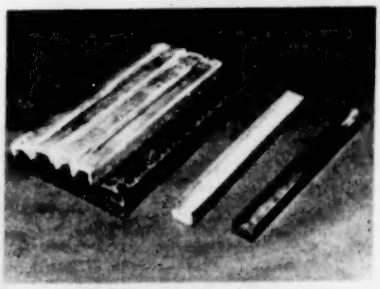
Preparation of standardized Salmonella suspensions. *Salmonella* stock cultures were maintained by monthly transplant on BBL trypticase soy agar slants, and working cultures were prepared by daily transfer in tubes of Difco tryptose broth. The tube contents (10-12 ml.) of a vigorously growing (4-hour old) culture were poured into 100 ml. of the broth and incubated for 18-20 hours at 37°C. The cells were harvested by centrifuging[4] and washing twice with sterile phosphate buffer pH 7.0 (M/15 KH_2PO_4 + M/15 Na_2HPO_4), the organisms were then resuspended in approximately 200 ml. of the buffer in a sterile one-liter Erlenmeyer flask containing glass beads and were stored in the refrigerator. A most probable number (MPN) count was performed on the suspension by preparing consecutive decimal dilutions in buffer and subculturing 1-ml. aliquots in quintuplicate tubes of tryptose broth. The seeded tubes were incubated for 24 hours at 37°C. and the bacterial populations estimated by employing McCrady's tables of most probable numbers *(10)*. The refrigerated suspensions used for thermal death time determinations were employed within 3 days of harvesting, although the MPN counts of these organisms remained constant for at least one week.

Experimental procedure for determination of thermal resistance. The stock suspension of the test organism was suitably diluted in neutral phosphate buffer so that a 5- to 20-ml. quantity of the final dilution, when added to the weighed, sterile egg pulp, provided a viable cell concentration of approximately 100,000 per ml. This cell load was selected to represent *Salmonella* populations ten-fold higher than those found by this Laboratory (unpublished data) in commercially broken-out liquid egg. After the inoculum had been introduced the melange was mixed for 15 minutes by means of an

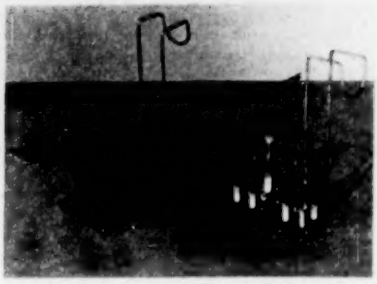
[4]During extremely hot summer weather the excessive heat generated while centrifuging resulted in cell suspensions of apparent lowered heat resistance. It was, therefore, found advisable to use a refrigerated centrifuge (10°C.) under these conditions.

electrically-driven stirrer. The substrate was dispensed aseptically into sterile, unplugged 10- × 75-mm. pyrex tubes (TDT tubes) by means of an automatic pipetting machine adjusted to deliver 1.1-ml. aliquots (Figure 1). The tubes were protected from outside contamination by sterile aluminum covers padded on the inside with cotton and gauze; the covers were made to slide over the tubes, momentarily exposing 2 tubes at a time for the pipetting operation (Figure 1). The filling operation required

A. Aseptic Tube Filling Operation.



B. Covers for protecting TDT tubes in rack.



C. Heating baskets showing sealed TDT tubes with substrate.

Figure 1. Apparatus used for filling and holding thermal death time tubes.

about 15 minutes. The TDT tubes were then sealed in an oxy-gas flame and transferred to specially constructed wire baskets, each basket containing 10 filled tubes (Figure 1). The inoculated egg was kept below the growth temperature range of the organism by holding the filled tubes in ice-water during the 2.5-3 hours of filling and sealing. The baskets were stored in a refrigerator at 39-41°F. until ready for processing, then were removed from the refrigerator, and 5 or 6 racks were simultaneously plunged into a heated glycerol bath maintained at a temperature constant to ± 0.25°F. Timing was begun at the moment of submersion. At selected intervals of time individual racks were withdrawn from the bath and immersed immediately into a bath of running tap water. The heating times employed were arrived at by performing preliminary three-replicate tube runs in order to locate the limits of survival of each organism within the pasteurization range used, 130-145°F.(54.4-62.8°C.). The sealed pasteurized TDT tubes were then incubated for 5 days at 37°C., and were opened by scoring the glass with a motor-driven carborundum cutting wheel and applying a flame to the mark. The presence of bacterial survivors was detected by subculturing one loopful of melange into tryptose broth tubes and examining for turbidity after incubation at 37°C. for 48 hours. Of several methods tested for the recovery of heated cells this technique was found to yield the largest number of positive samples. When endpoints of survival

showed fewer than 4 positive tubes out of 10, cultures were confirmed on Difco triple sugar iron agar and typed with the appropriate *Salmonella* group antisera in order to rule out possible contaminating organisms.

For the purpose of checking the cell load in the inoculated egg 3 representative 1.1-ml. samples of the melange were delivered into 18- × 150-mm. dry, sterile tubes during the machine-filling of the TDT tubes. The control samples were held under the same environmental conditions as the TDT tubes until the completion of the sealing operation. They were appropriately diluted with phosphate buffer and the MPN count determined by the dilution to extinction technique. The results of the triplicate counts were averaged.

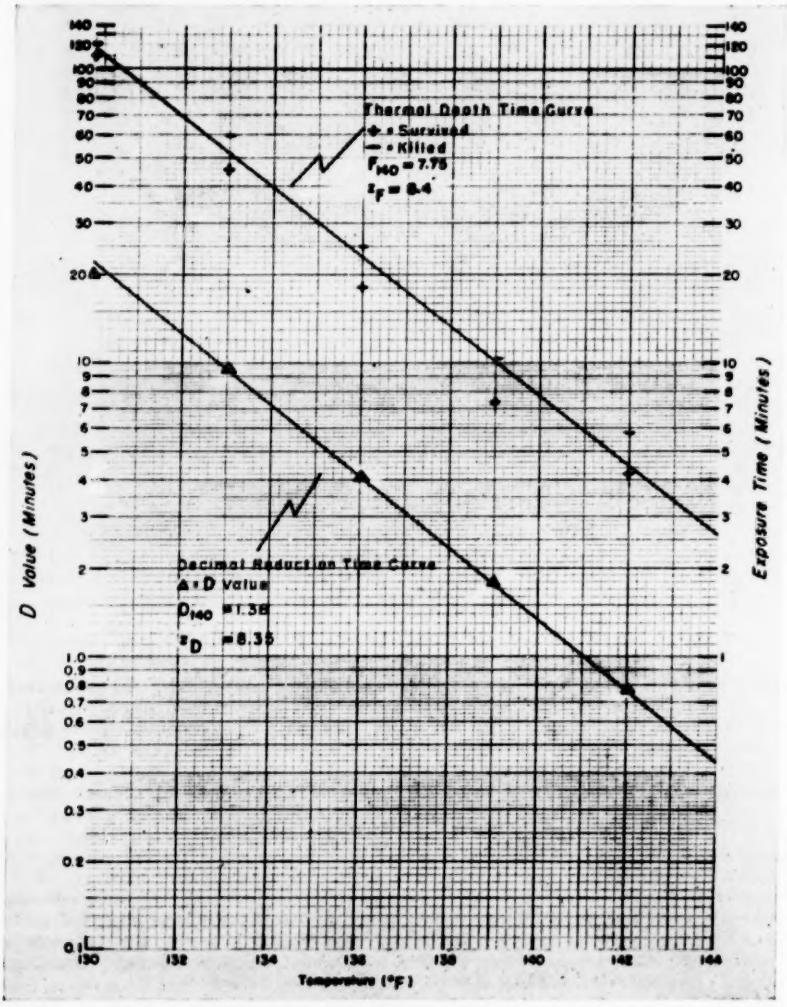


Figure 2. Heat resistance of Salmonella oranienburg 1011 in liquid whole egg. pH 5.5; approximate cell load = 0.88 × 10^5 per tube.

Determination of rate of heat penetration. The sample of sterile egg reserved for the heat lag measurements was diluted with a quantity of phosphate buffer equivalent to the inoculum used in the thermal resistance study. Portions of 1.1 ml. of the egg were pipetted into TDT tubes. Each tube was provided with a thermocouple similar to that described by Sognefest and Benjamin *(40)*. The filled tubes were first cooled in ice-water at 39-41°F. for at least 15 minutes, and after the copper-constantan thermocouple leads were connected, single tubes were submerged in the constant-temperature bath. Timing began at the instant of immersion and millivolt readings were taken at 10-second intervals with a Brown portable potentiometer. When the tubes reached maximum constant voltage equivalent to that obtained by a control thermocouple in the bath, they were transferred to a bath of running tap water and the cooling rate was recorded every 5 seconds. At each bath temperature selected, 4 replicate samples were measured and their millivolt averages per time interval were converted to °F. Ten separate heat penetration experiments were performed within the pasteurization range 130-145°F.(54.4-62.8°C.) in increments of 3°F.

A heat penetration curve for each bath temperature (Figure 3) was then drawn on semilog paper which had been rotated 180°. The rotation of the paper provides a log scale which increases from top to bottom, the top line being given an arbitrary value of 0.1°F. below bath temperature, since the potentiometer employed could not detect temperature differences less than 0.1°F. Time was plotted on the linear scale, and the difference between bath temperature (BT) and substrate temperature (ST)* on the log scale. This type of heat penetration curve produced straight lines for both the heating and cooling portions of the plot, since the substrate does not exhibit a sharp break in viscosity within the temperature range employed. It was observed that each of the resulting curves reached 0.1°F. below the different bath temperatures at substantially the same time, and that their slopes were practically identical. The straight line curve on semilog paper was preferred to the curvilinear plot on linear paper because: (a) the time of come-up to 0.1°F. below bath temperature, a value necessary for thermal lag corrections, is more accurately located on such a straight line graph, and (b) the heating and cooling properties of a substrate can readily be characterized by a point and a slope *(2)*.

Calculation of thermal processes. A. *Correction factor for the construction of thermal resistance curves.* Since instantaneous heating to bath temperature does not occur when sealed tubes are plunged into the bath nor does instantaneous cooling follow the removal of the tubes to cold water, it is necessary to take into account 2 factors for correcting the observed time of exposure before a true thermal process can be calculated. One corrective factor is the heating lag, which is the time required for the substrate to reach bath temperature, followed by cooling to a non-lethal temperature. Inasmuch as thermal destruction of the bacteria proceeds while the substrate is being heated to bath temperature and continued destruction occurs during cooling, a second time correction must be made to account for this lethal effect on the cell suspension. The correction factor, therefore, takes into account both the thermal lag and the lethality during lag, which was calculated in the following manner:

A thermal death time curve of the experimental data secured was constructed on semilog coordinates by plotting temperature linearly and the end points of survival-kill logarithmically (Figure 2). The curve was fitted as a straight line to best represent the end points of survival and kill.

Each point on the heating and cooling curve provides a certain lethal rate value, or some degree of bacterial destruction *(7)*. This value was calculated by determining the killing time on the F curve corresponding to the desired temperature on the thermal lag plot, and taking its reciprocal (i.e., lethal rate = 1/killing time). A sufficient number of such values were obtained so that when lethal rates were plotted on linear coordinates against the times required to attain the temperatures chosen on the heat penetration curve, a smooth lethality curve was produced. Such a plot (Figure 4) was prepared for each bath temperature studied. The area under each lethality curve (A), measured with a planimeter, represented lethality occurring during actual heating and cooling of the bacterial suspension. A rectangle (B) was then constructed on

---

*BT—ST is analogous to RT—CT in canning practice, where RT is the retort temperature and CT is the can temperature.

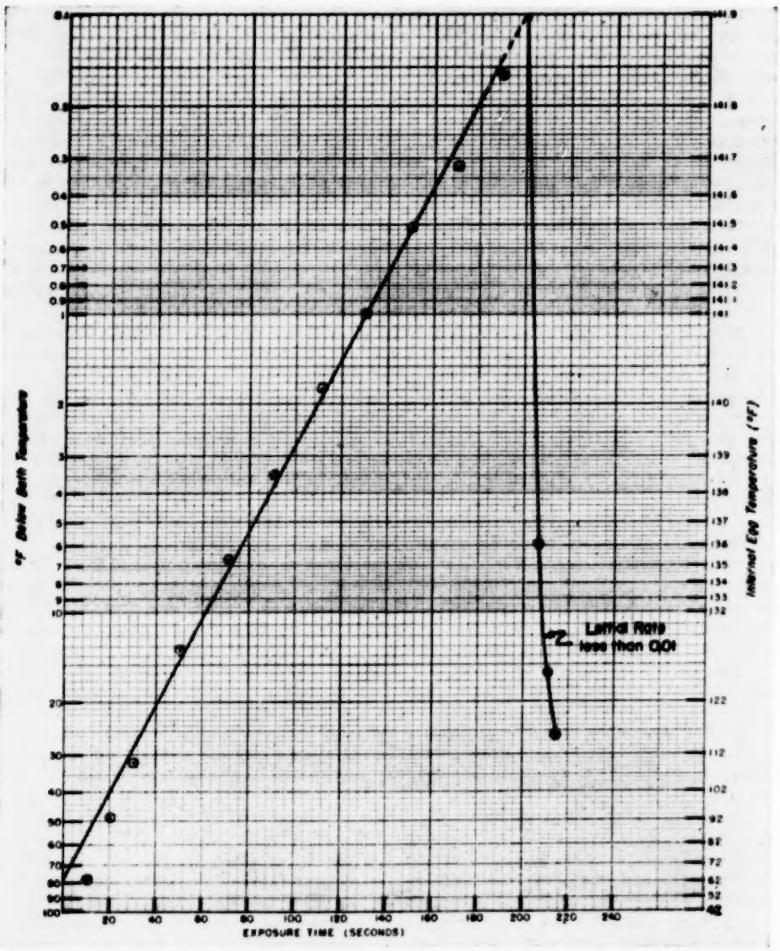


Figure 3. Rate of heat penetration into 1.1 ml. liquid egg pH 5.5 contained in 10 × 75 mm. pyrex tubes. Average of 40 measurements for bath temperature 142°F. (61.1°C.).

the lethality curve coordinates with the ordinate equal to the highest lethal rate of the given bath temperature and the abscissa corresponding to one minute of heating. The area of this rectangle represented lethality occurring during a treatment involving instantaneous heating to bath temperature, holding for one minute, and instantaneous cooling to a non-lethal temperature. The ratio of A/B for a given bath temperature provided the calculated lethality correction factor representing additional minutes of lethal exposure occurring during come-up and come-down.

Therefore, the heat exposure time corrected for thermal lag and for lethality during lag is expressed by the following formula:

Exposure time (corrected) = Total exposure time in bath — Thermal lag time + Lethality due to time of come-up and come-down.

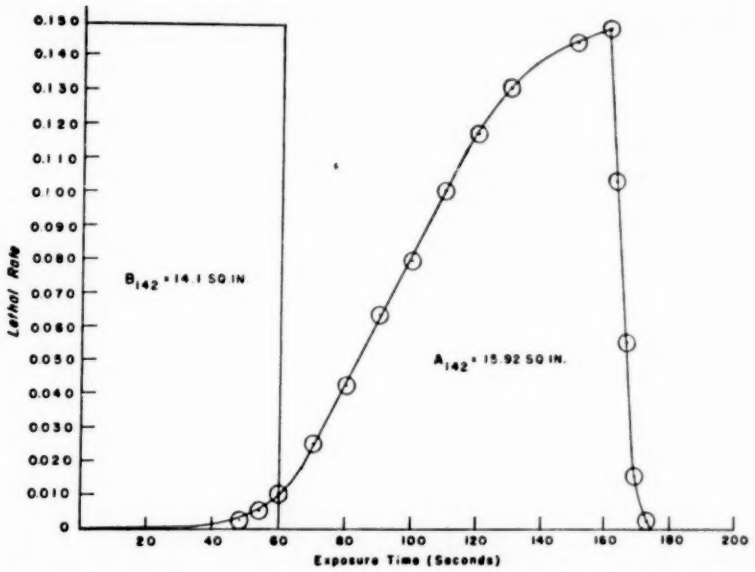


Figure 4. Lethality curve for Salmonella oranienburg 1011 in liquid whole egg. pH 5.5; approximate cell load = 0.88 × 10^6 per tube.

The differences between heat penetration times and lethalities produced a mean correction factor of 1.7 minutes. This value, deducted from the total exposure time in the bath, gave end points of survival-kill corrected for thermal lag plus lethality due to this lag. A corrected thermal death time curve was then constructed and was defined in terms of its F_{140} and z_F values (Figure 2). While a correction value of 1.7 often may appear relatively large at the upper temperature, 140-142°F., this constituted only a very minor portion of the total exposure times below that temperature range. Hence, in plotting thermal death time curves somewhat greater weight was placed on end points of destruction at the lower temperatures.

B. *Computation of D Values.* D values were calculated by Schmidt's method as described by Reynolds *et al. (36)*. The constant 0.69 has been substituted for 0.5 in accordance with a suggestion by Schmidt *(38)*. The equation therefore becomes:

$$D = \frac{L.D._{50}}{\log \text{ initial number of organisms per tube} - \log 0.69}$$

where $L.D._{50}$ is the time in minutes on the probability curve where the probability of a tube showing growth or being sterile is equal to 0.5. The decimal reduction time curve was expressed in terms of D_{140} and z_D values (Figure 2).

RESULTS AND DISCUSSION

In accordance with the methods described above, thermal resistance studies in liquid whole egg were conducted using 10 *Salmonella* cultures representing seven serotypes as well as 4 strains of one type. In one series of experiments the heat resistance was determined in liquid whole egg adjusted to pH 5.5. Another group of experiments was undertaken in which the influence of pH on thermal resistance was closely followed

throughout the range pH 5.5 to 8.5 in the investigation of one highly resistant strain. Selected cultures were employed in a third series measuring the thermal tolerance in egg at pH 8.0. Variations in heat resistance which occur in different strains of a single serotype were demonstrated in a fourth set of measurements. All experimental runs were repeated, the replicate runs showing good agreement, in general.

Thermal resistance of Salmonella in egg at pH 5.5. A summary of thermal death time data for *Salmonella pullorum*, *S. oranienburg*, *S. montevideo*, *S. meleagridis*, *S. worthington*, *S. typhimurium*, and *S. senftenberg* is presented in Table 1. The results are expressed in terms of F_{140} and z_F for the conventionally used thermal death time curves. In addition, corresponding values for D_{140} and z_D are tabulated to represent the decimal reduction time-temperature relationship associated with the "phantom" thermal death time curve of Ball *(4)*.

It will be noted that the slopes (z_F) of the F curves for 6 of the 7 types of *Salmonella* are approximately the same (7.4 to 8.3). Only in the case of *S. senftenberg* 775W is the z_F value greater (12.3) and, consequently, the thermal death time line is somewhat less steep.

The F_{140} values of the 7 cultures in acidified egg (pH 5.5) ranged from a low of 2.9 minutes for *S. pullorum* 13117 to an extremely high value of 83.6 minutes for *S. senftenberg* 775W, with a group of 4 cultures (*S. meleagridis* 5485, *S. montevideo* 1162, *S. oranienburg* 1011, and *S. worthington*

TABLE 1

Heat resistance of Salmonella serotypes in liquid whole egg, pH 5.45-5.55 (Approximate cell load = 100,000 per tube)

Test organism	F_{140} minutes	z_F °F.	D_{140} minutes	z_D °F.
S. pullorum 13117	2.80	7.60	0.40	7.40
S. pullorum 13117	2.95	7.20	0.40	7.50
Average	2.9	7.4	0.40	7.5
S. worthington 20A2	6.00	7.25	1.00	7.10
S. worthington 20A2	7.70	8.15	1.20	8.30
Average	6.9	7.7	1.1	7.7
S. oranienburg 1011	7.35	7.85	1.10	7.80
S. oranienburg 1011	7.75	8.40	1.40	8.35
Average	7.6	8.1	1.3	8.1
S. montevideo 1162	7.55	7.90	1.20	7.90
S. montevideo 1162	10.25	8.45	1.60	8.40
Average	8.9	8.2	1.4	8.2
S. meleagridis 5485	8.60	8.05	1.60	7.70
S. meleagridis 5485	9.35	7.65	1.60	7.90
Average	9.0	7.9	1.6	7.8
S. typhimurium 81	13.60	7.80	2.10	7.80
S. typhimurium 81	16.60	8.75	2.30	7.85
Average	15.1	8.3	2.2	7.8
S. senftenberg 775W	84.00	14.30	7.00	9.80
S. senftenberg 775W	86.75	11.85	10.50	9.80
S. senftenberg 775W	80.00	10.75	11.00	10.90
Average	83.6	12.3	9.5	10.2

20A2) showing a medium resistance between 6.9 and 9.0 minutes. The F_{140} value (15.1 minutes) of *S. typhimurium* 81 was found to lie between the medium group of organisms and *S. senftenberg* 775W.

The decimal reduction time curve parallels the conventional thermal death time curve, as is clearly indicated by the close agreement between values for z_F and z_D. With the exception of the highly resistant strain, *S. senftenberg* 775W, it is seen that the slopes of the D_{140} curves vary only from 7.5 to 8.2. When one contrasts the z value of approximately 8 for these vegetative cells with the typical z values of 18-20 for bacterial spores such as *Clostridium botulinum* or P.A. 3679, one realizes the relative steepness of the thermal resistance curve associated with the *Salmonellae*. The low z value of this group of organisms indicates a high degree of sensitivity to small changes in heating temperatures at a fixed time; thus, a small decrease in temperature could permit the survival of a significant number of bacteria with a resultant inadequately pasteurized product. The maintenance of close temperature and time controls are therefore of great importance in egg pasteurization in order to produce a *Salmonella*-free pulp.

Influence of pH on thermal resistance of Salmonella. When *Salmonella* organisms of exceptionally high thermal resistance—for example, *S. senftenberg* 775W ($F_{140} = 83.6$)—are encountered in egg pulp acidified to pH 5.5, the relatively short holding times for commercial pasteurization would obviously be inadequate for the elimination of such strains. In order to find more satisfactory conditions for pasteurization, which would destroy cultures of extreme resistance, the investigation of pH influence on F and D values was undertaken.

The importance of pH as a factor in bacterial heat resistance has long been recognized. Investigations of bacterial spore resistance by Weiss *(47)*, Esty and Meyer *(13)*, and others have shown that a pH region exists where thermal death time attains a maximum, and that beyond that region increases in acidity or alkalinity result in lowered tolerance to heat. Although reports of similar systematic studies over a wide pH range are lacking for non-sporing bacteria, limited evidence is available that the same pH-heat resistance relationship is valid for vegetative cells. Thus, at pH levels on the alkaline side for liquid whole egg, Greco *(19)* showed that *Escherichia coli* was more resistant at pH 6.6 than at 7.5, and Winter *(49)*, employing *Salmonella*, found a similar lowering of resistance when the pH of the egg was changed from 5.5 to 7.2. On the acid side of the pH-resistance curve studies by Beamer and Tanner *(6)* with *Salmonella* in nutrient broth showed that lowering the pH from 7.05 to 3.63 also resulted in reduced heat resistance.

In the present investigation experiments were designed to study *Salmonella* heat resistance in liquid whole egg over the critical pH range from 5.5 to 8.5. Data in Table 2 show typical results for *S. senftenberg* 775W. It is seen that the extreme heat tolerance of 83.6 minutes at pH 5.5 can be sharply reduced to 3.1 minutes by adjusting the pH to 8.5 and that the slopes of the F and D curves tend to steepen slowly with the increase in pH. The change in F_{140} values is a continuous function of pH in the range between pH 6.1 and 8.5, and the relationship is graphically illustrated in Figure 5. There appears to be a plateau of maximum ther-

TABLE 2

Influence of pH on the heat resistance of S. senftenberg 775W in liquid whole egg (Approximate cell load = 100,000 per tube)

pH of egg	F_{140} minutes	z_F °F.	D_{140} minutes	z_D °F.
5.45	84.00	14.30	7.00	9.80
5.45	86.75	11.85	10.50	9.80
5.55	80.00	10.75	11.00	10.90
Average	83.6	12.3	9.5	10.2
6.1	88.75	11.95	11.00	10.00
6.2	82.75	10.40	10.50	9.80
Average	85.8	11.2	10.8	9.9
6.7	62.25	11.95	9.50	12.60
6.6	63.60	11.80	8.50	11.50
Average	62.9	11.9	9.0	12.1
7.35	48.00	11.10	5.20	10.60
7.35	48.00	10.30	4.00	9.40
Average	48.0	10.7	4.6	9.5
8.0	10.25	8.60	1.20	8.70
8.0	11.70	9.95	1.70	10.30
Average	11.0	9.3	1.5	9.5
8.50	2.85	7.10	0.36	7.45
8.55	3.30	8.70	0.36	8.10
Average	3.1	7.9	0.36	7.8

mal resistance between pH 5.5 and 6.1, a sharp drop in F_{140} value between pH 6.1 and 8.0, and a tendency to level off in the neighborhood of pH 8.5. Experiments to determine the susceptibility of the organism to pH 9.0 were discontinued because the melange under these alkaline conditions suffered marked visible degradation during heat treatment as evidenced by darkening with loss of viscosity. Considering the general shape of the pH-thermal death time curves for other micro-organisms *(13)* and the shape of the curves in Figure 5, it would be reasonable to expect lowered heat resistance for *S. senftenberg* 775W at pH levels somewhere below 5.5. Such a possibility was not investigated, since pH values less than 5.5 approach too closely to the coagulating point of the egg albumen.

A non-linearity in thermal resistance curves, which was observed in only two sets of experiments, is worthy of mention. These anomalies appeared at pH 6.6 and at 7.35. Although the deviations from linearity were small, they were reproduced in repeated tests. It was also noted that in this pH region—in the steep portion of the pH curve shown in Figure 5—the alkalinity of the egg increased after pasteurization by as much as 0.6 pH unit, whereas little or no such changes occurred in the heated melange on either side of this pH range. It is consequently believed that the anomalies resulted from a disproportionate shift in thermal resistance due to changes in pH during the heating of the liquid egg. To fit the thermal resistance data to straight line functions in these two cases, the method of least squares was employed.

From Figure 5, it is obvious that the pH of the egg pulp is a critical factor in the heat stability of *S. senftenberg* 775W, and data shown in

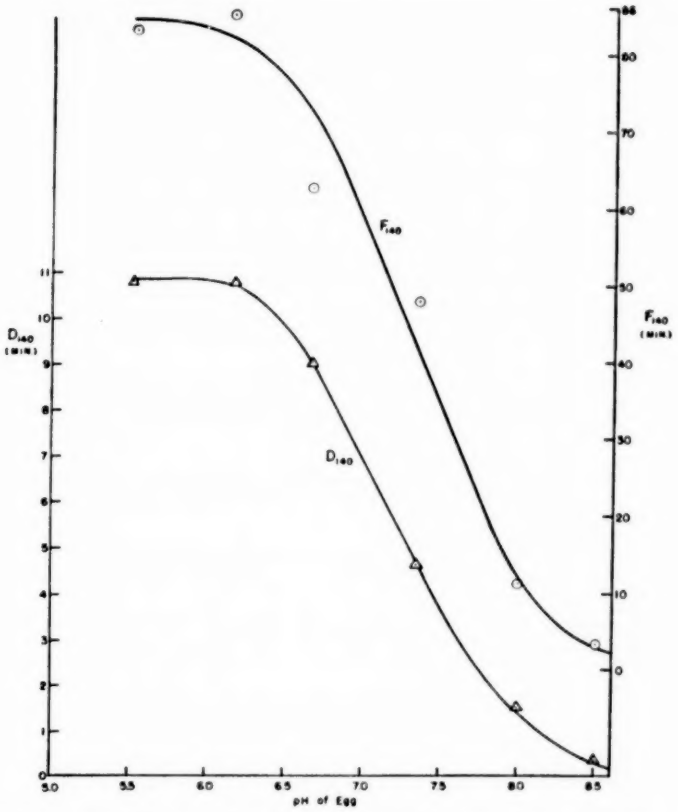


Figure 5. Influence of pH on the heat resistance of Salmonella senftenberg 775W in liquid whole egg. Approximate cell load = 1×10^5 per tube.

Table 3 for other organisms in the *Salmonella* genus supply additional supporting evidence. Lower F_{140} values in pH 8.0 egg than in the pH 5.5 product were observed for *S. pullorum*, *S. montevideo*, *S. typhimurium*, and *S. worthington*. The rate of destruction of these cultures was approximately three times more rapid in the alkaline melange, while total killing of *S. senftenberg* was accomplished even more rapidly at the higher pH level.

The observations of Winter *(49)* and Osborne, Straka, and Lineweaver *(31)*, that *Salmonellae* survive heating for longer periods in acidified egg than in unacidified egg, have been confirmed. However, comparisons of heat resistances with these investigators, expressed as F or D values, could not be made.

Heat resistance of different strains of S. senftenberg in liquid whole egg, pH 8.0. The extreme tolerance to heat possessed by *S. senftenberg* 775W is not a common property of the serological type. In Table 4 are

TABLE 3

Heat resistance of selected Salmonella serotypes as influenced by the pH of liquid whole egg

(Approximate cell load = 100,000 per tube)

Test organism	pH of Liquid Whole Egg							
	pH 5.5				pH 8.0			
	F_{140} minutes	z_F °F.	D_{140} minutes	z_D °F.	F_{140} minutes	z_F °F.	D_{140} minutes	z_D °F.
S. pullorum 13117	2.80	7.60	0.40	7.40	1.00	8.15	0.18	8.20
S. pullorum 13117	2.95	7.20	0.40	7.50	0.79	7.40	0.10	7.10
Average	2.9	7.4	0.40	7.5	0.9	7.8	0.14	7.7
S. worthington 20A2	6.00	7.25	1.00	7.10	2.5[1]	7.35	0.43[1]	7.40
S. worthington 20A2	7.70	8.15	1.20	8.30	.....	.....	.....	.....
Average	6.9	7.7	1.1	7.7	.....	.....	.....	.....
S. montevideo 1162	7.55	7.90	1.20	7.90	3.10	9.30	0.48	9.80
S. montevideo 1162	10.25	8.45	1.60	8.40	3.45	8.45	0.62	8.50
Average	8.9	8.2	1.4	8.2	3.3	8.9	0.55	9.2
S. typhimurium 81	13.60	7.80	2.10	7.80	3.25	8.50	0.50	8.90
S. typhimurium 81	16.60	8.75	2.30	7.85	4.75	8.15	0.59	7.85
Average	15.1	8.3	2.2	7.8	4.0	8.3	0.55	8.4
S. senftenberg 775W	84.00	14.30	7.00	9.80				
S. senftenberg 775W	86.75	11.85	10.50	9.80	10.25	8.60	1.20	8.70
S. senftenberg 775W	80.00	10.75	11.00	10.90	11.70	9.95	1.70	10.30
Average	83.6	12.3	9.5	10.2	11.0	9.3	1.5	9.5

[1]pH of egg = 7.6.

presented thermal resistance data of 4 strains of *S. senftenberg* isolated from egg products. It will be noted that the exceedingly high F_{140} value indicated by strain 775W is unusual. The three other strains tested show heat susceptibilities similar to that of *S. montevideo* (Table 3), an organism representing the degree of heat resistance common to the other types reported in this investigation.

Relationship between F and D values. In line with current developments in thermal resistance theory, and for the purposes of comparison, D_{140} values are presented in Tables 1-4. Inspection of Table 1 shows that, as in the case of the F_{140} data, *S. pullorum* was the least heat resistant of the cultures tested ($D_{140} = 0.4$) while *S. senftenberg* 775W was the most heat stable ($D_{140} = 9.5$). *S. worthington, S. oranienburg, S. montevideo,*

TABLE 4

Heat resistance of different strains of S. senftenberg in liquid whole egg, pH 8.0 (Approximate cell load = 100,000 per tube)

Strain number	F_{140} minutes	z_F °F.	D_{140} minutes	z_D °F.
775W	10.25	8.60	1.20	8.70
775W	11.70	9.95	1.70	10.30
Average	11.0	9.3	1.5	9.5
523	2.45	8.10	0.33	7.90
523	2.65	8.10	0.37	8.00
Average	2.6	8.1	0.35	8.0
2623	3.45	8.60	0.50	8.50
2623	2.50	8.10	0.39	8.00
Average	3.0	8.4	0.45	8.3
3252	1.75	7.90	0.29	7.75
3252	2.55	8.10	0.42	8.10
Average	2.2	8.0	0.36	7.9

and *S. meleagridis* were of medium tolerance to heat ($D_{140} = 1.1$ to 1.6), and *S. typhimurium* was somewhat more resistant than the medium group of four ($D_{140} = 2.2$).

The influence of pH on the thermal resistance of *S. senftenberg* 775W (Table 2) is again clearly demonstrated in the decrease of the D_{140} value (from 10.8 to 0.36) with increasing alkalinity of the egg substrate. Other types of *Salmonella* are similarly affected by pH changes (Table 3).

That strain 775W of *S. senftenberg* is not representative of that serotype is shown by the low D_{140} values (0.36 to 0.45) of three other *S. senftenberg* cultures originally isolated from egg products (Table 4).

Limited unpublished data exists *(38)* which indicate a direct relationship between F and D values in the case of sporeforming bacteria. No such information is available for non-sporulating organisms. It would be of distinct value if a direct relationship between F and D could be found in this investigation, so that one given value could be predicted in terms of the other. An attempt was made to find such a relationship.

A statistical examination of the thermal resistance data of 25 runs (excluding those obtained for *S. senftenberg* 775W) yielded a high degree of linear correlation ($r = 0.99$) between the F_{140} and D_{140} values, with a

standard error from the line of regression of only 0.55. The relationship between F and D can be expressed as:

$$F_{140} = 7.23\ D_{140} - 0.32 \pm 0.55.$$

This formula predicts that 67% of the cases for F should fall within $\pm$ 0.55 minutes of the true value. By doubling the standard error, 95% of the predictions for F should lie within ± 1.1 minutes of the true value. The equation then becomes:

$$F_{140} = 7.23\ D_{140} - 0.32 \pm 1.1$$

Because the initial *Salmonella* population was relatively constant in each of the 25 cases, the value of F in the above expression was independent of the original cell load.

Application of D values to pasteurization. A measure of the adequacy of any egg pasteurization treatment is the statistical frequency of detecting *Salmonella* survivors in random samples selected to represent typical lots of commercially pasteurized eggs. It can be calculated that when the number of survivors is of the order of 0.01 per g., the probability of detecting *Salmonella* with a standard procedure of analysis is about 11 samples in 100. If, on the other hand, the concentration of survivors is reduced to 0.001 per g., then the corresponding probability becomes approximately one chance in 100. The D value concept is particularly useful in choosing a level of heat processing to yield any given arbitrary degree of bacterial survival in the egg product.

The importance of the D value in establishing pasteurization conditions for egg may be illustrated by the following examples: Assuming an initial *Salmonella* concentration of 10^4 per g. in a 10,000-pound batch of liquid whole egg, the total *Salmonella* content of the lot would be equal to $10{,}000 \times 454 \times 10^4$, or 4.54×10^{10} *Salmonella*. Since D is the time, in minutes, required to destroy 90% of the initial bacterial load, and a $2 \times$ D treatment affords a 99% reduction, it is apparent that a $7 \times$ D treatment will provide a 99.99999% degree of destruction. Such a 7D pasteurization will reduce the level of survivors to only 0.00001% of the initial count, 4.54×10^{10}, which can be computed as 4.54×10^3 survivors. Distributed through the 10,000 pounds of liquid egg, these 4,540 *Salmonella* would offer a final concentration of 0.454 survivors per pound. This is equivalent to 0.001 *Salmonella* per g. and satisfies the probability considerations mentioned above.

It has recently been indicated by Goresline and co-workers *(18)* that pasteurization at 140°F. for 3 minutes may be a practical level for eliminating *Salmonella* from liquid whole egg. In their experiments commercial scale pasteurization tests were conducted using egg at its natural pH (7.5-7.9). The present data on F and D values shown in Tables 3 and 4 suggest that 3 minutes at 140°F. may be a marginal treatment and could result in an inadequately pasteurized pulp, even assuming the absence of extremely heat resistant strains such as *S. senftenberg* 775W. With egg at its natural pH it would appear that an F_{140} of approximately 4 should be employed for the destruction of the moderately resistant *Salmonella* organisms reported in this study. This could be equivalent to a treatment

just above 7D when D = 0.55. Even for the infrequently encountered strains possessing high resistance such as *S. senftenberg* 775W with a D_{140} value of 1.5, pasteurizing at 140°F. for 4 minutes would result in a degree of destruction greater than 99.5% of the initial contamination.

SUMMARY

The heat resistance in liquid whole egg of ten *Salmonella* strains was studied in the temperature range from 130 to 145°F. The organisms employed were originally isolated from spray-dried egg and represent seven serotypes; namely, *S. pullorum*, *S. montevideo*, *S. oranienburg*, *S. worthington*, *S. meleagridis*, *S. typhimurium*, and *S. senftenberg*.

Thermal death time curves and decimal reduction time curves were obtained; F_{140} and D_{140}, points on the curves, were adopted for designating the heat resistance of these bacteria.

It has been shown that the pH of liquid egg is an important factor in the heat resistance of *Salmonella*. The F_{140} and D_{140} values change as a continuous function with pH in the range between pH 6.1 and 8.5.

The slopes of the thermal death time curve and the decimal reduction time curve agreed closely. Average *z* values for *Salmonella* organisms in liquid egg ranged from 7.5 to 8.5.

The majority of the *Salmonella* organisms tested showed F_{140} values ranging from 7 to 9 minutes at pH 5.5 and F_{140} values of 2.0 to 3.5 minutes at pH 8.0. D_{140} values of 1.0 to 1.5 were lowered to 0.4 to 0.6 minute with a corresponding increase in egg pH.

Marked differences in heat resistance were found among individual strains of the serotype *S. senftenberg*. Strain 775W was the most resistant of the *Salmonella* studied, its F_{140} value being 11.0 minutes at pH 8.0, whereas three other strains showed F_{140} values of 2 to 3 minutes at the same pH.

A relationship between F and D values has been presented, and the application of D values to pasteurization of egg discussed.

Acknowledgment

The authors gratefully acknowledge the advice and assistance of Dr. Clarence F. Schmidt, Continental Can Company, regarding thermal process calculations, and for his reading and helpful criticism of the manuscript. We are also indebted to Mr. Kenneth R. Wood, QM Food and Container Institute for the Armed Forces, for his statistical analysis of the data.

LITERATURE CITED

1. American Can Company. Modern Thermal Process Determination. *The Canned Food Reference Manual*, 3rd ed., 1947. American Can Company, New York, N. Y.
2. Ball, C. O. Thermal process time for canned foods. *Bull. Nat. Research Council*, **7**, Part 1, No. 37, 1-76 (1923).
3. Ball, C. O. Mathematical solution of problems on thermal processing of canned foods. Univ. of Calif. *Pubs. in Public Health*, **1**, 15-245 (1928).
4. Ball, C. O. Short-time pasteurization of milk. *Ind. Eng. Chem.*, **35**, 71-84 (1943).
5. Baselt, F. C. Pasteurization. Communications, *Master Brewers Assoc. of America*, **10**, No. 11-12, 13-16 (1949).
6. Beamer, P. R., and Tanner, F. W. Resistance of non-spore-forming bacteria to heat. *Zentr. Bakt. Parasitenk., Abt. II*, **100**, 81-98 (1939).
7. Bigelow, W. D., Bohart, G. S., Richardson, A. C., and Ball, C. O. Heat penetration in processing canned foods. *Nat. Canners' Assoc. Bull.* **16L**, (1920).

8. BIGELOW, W. D., AND ESTY, J. R. The thermal death-point in relation to time of typical thermophilic organisms. *J. Infectious Diseases*, **27**, 602-17 (1920).
9. BOGGS, M. M., AND FEVOLD, H. L. Dehydrated egg powders. Factors in palatability of stored powders. *Ind. Eng. Chem.*, **38**, 1075-79 (1946).
10. BUCHANAN, R. R., AND FULMER, E. I. *Physiology and Biochemistry of Bacteria.* Vol. I, p. 12, 1928, Williams and Wilkins Co., Baltimore, Md.
11. CONRAD, R. M. University of Denver (Personal communication, 1946), cited by Lightbody and Fevold (23).
12. DEL VECCHIO, H. W., DAYHARSH, C. A., AND BASELT, F. C. Thermal death time studies on beer spoilage organisms. I. *Am. Soc. Brewing Chemists Proc.*, 45-59 (1951).
13. ESTY, J. R., AND MEYER, K. F. The heat resistance of the spores of *B. botulinus* and allied anaerobes. XI. *J. Infectious Diseases*, **31**, 650-63 (1922).
14. ESTY, J. R., AND WILLIAMS, C. C. Heat resistance studies. I. A new method for the determination of heat resistance of bacterial spores. *J. Infectious Diseases*, **34**, 516-28 (1924).
15. GILLESPY, T. G. The heat resistance of the spores of thermophilic bacteria. I. Introductory. *Ann. Rept. Fruit and Veg. Presvn. Research Sta.*, Campden, 40-51 (1946).
16. GILLESPY, T. G. The heat resistance of the spores of thermophilic bacteria. III. Thermophilic anaerobes. *Ann. Rept. Fruit and Veg. Presvn. Research Sta.*, Campden, 34-43 (1948).
17. GILLESPY, T. G. Estimation of sterilizing value of processes as applied to canned foods. I. Packs heating by conduction. *J. Sci. Food Agric.*, **2**, 107-25 (1951).
18. GORESLINE, H. E., HAYES, K. M., MOSER, R. E., JR., HOWE, M. E., AND DREWNIAK, E. E. Pasteurization of liquid whole egg under commercial conditions to eliminate *Salmonella*. U. S. Dept. of Agric. *Circular No. 897*, October (1951).
19. GRECO, P. A. The pasteurization of liquid whole egg. (Ph.D. Thesis, Iowa State College, Ames, Iowa, 1943.)
20. HICKS, E. W. On the evaluation of canning processes. *Food Technol.*, **5**, 134-42 (1951).
21. HICKS, E. W. Some implications of recent theoretical work on canning processes. *Food Technol.*, **6**, 175-78 (1952).
22. KATZIN, L. I., SANDHOLZER, L. A., AND STRONG, M. E. Application of the decimal reduction time principle to a study of the resistance of coliform bacteria to pasteurization. *J. Bact.*, **45**, 265-72 (1943).
23. LIGHTBODY, H. D., AND FEVOLD, H. L. Biochemical factors influencing the shelf life of dried whole eggs and means for their control. *Advances in Food Research*, 1, 1948. Academic Press, Inc., New York, N. Y.
24. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. I. Pathogenicity of strains of *Salmonella meleagridis* and *Salmonella anatum* obtained from spray-dried whole egg. *J. Infectious Diseases*, **88**, 278-89 (1951).
25. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. II. Immunity studies following experimental illness with *Salmonella meleagridis* and *Salmonella anatum*. *J. Immunol.*, **66**, 595-608 (1951).
26. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. III. Pathogenicity of strains of *Salmonella newport*, *Salmonella derby* and *Salmonella bareilly* obtained from spray-dried whole egg. *J. Infectious Diseases*, **89**, 209-13 (1951).
27. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. IV. Pathogenicity of strains of *Salmonella pullorum* obtained from spray-dried whole egg. *J. Infectious Diseases*, **89**, 259-65 (1951).
28. U. S. Army Quartermaster Corps, Military Specification MIL-E-10006 (QMC), 12 September 1949. Egg, Stabilized, Dehydrated.
29. OLSON, J. C., JR., MACY, H., AND HALVORSON, H. O. Thermal death-time studies of coliform bacteria in milk. Univ. of Minn. Agr. Exp. Sta. *Tech. Bull. 202*, June 1952.
30. OLSON, F. C. W., AND STEVENS, H. P. Thermal processing of canned foods in tin containers. II. Nomograms for graphic calculation of thermal processes for non-acid canned foods exhibiting straight-line semilogarithmic heating curves. *Food Research*, **4**, 1-20 (1939).

31. Osborne, W. W., Straka, R. P., and Lineweaver, H. Heat resistance of several strains of *Salmonella* in liquid whole egg, egg white and egg yolk. *Food Technol.*, **6** (5), Abstracts, 12th Annual Meeting, I.F.T., Paper 73 (1952).
32. Patashnik, M. A simplified procedure for thermal process evaluation. *Food Technol.*, **7**, 1-6 (1953).
33. Pearce, J. A., Reid, M., and Cook, W. H. Dried whole egg powder. XVIII. The keeping quality of acidulated, gas-packed powders of low moisture content. *Can. J. Research*, **24F**, 39-46 (1946).
34. Pflug, I. J., and Esselen, W. B. Development and application of an apparatus for study of thermal resistance of bacterial spores and thiamine at temperatures above 250°F. *Food Technol.*, **7**, 237-41 (1953).
35. Quartermaster Food and Container Institute. Unpublished reports on storage studies.
36. Reynolds, H., Kaplan, A. M., Spenser, F. B., and Lichtenstein, H. Thermal destruction of Cameron's putrefactive anaerobe 3679 in food substrates. *Food Research*, **17**, 153-67 (1952).
37. Schmidt, C. F. A method for the determination of the thermal resistance of bacterial spores. *J. Bact.*, **59**, 433-37 (1950).
38. Schmidt, C. F. A procedure for the interpretation of thermal death time data. (Personal communication, 1952.)
39. Schultz, O. T., and Olson, F. C. W. Thermal processing of canned foods in tin containers. III. Recent improvements in the general method of thermal process calculations—a special coordinate paper and methods of converting initial and retort temperatures. *Food Research*, **5**, 399-407 (1940).
40. Sognefest, P., and Benjamin, H. A. Heating lag in thermal death-time cans and tubes. *Food Research*, **9**, 234-43 (1944).
41. Solowey, M., Sutton, R. R., and Calesnick, E. J. Heat resistance of *Salmonella* organisms isolated from spray-dried whole egg powder. *Food Technol.*, **2**, 9-14 (1948).
42. Stewart, G. F., Best, L. R., and Lowe, B. A study of some factors affecting the storage changes in spray-dried egg products. *Proc. Inst. Food Technol.*, 77-89 (1943).
43. Stumbo, C. R. A technique for studying resistance of bacterial spores to temperatures in the higher range. *Food Technol.*, **2**, 228-40 (1948).
44. Stumbo, C. R. Bacteriological considerations relating to process evaluation. *Food Technol.*, **2**, 115-32 (1948).
45. Stumbo, C. R. Further considerations relating to evaluation of thermal processes for foods. *Food Technol.*, **3**, 126-31 (1949).
46. Stumbo, C. R., Murphy, J. R., and Cochran, J. Nature of thermal death time curves for P.A. 3679 and *Clostridium botulinum*. *Food Technol.*, **4**, 321-26 (1950).
47. Weiss, H. The heat resistance of spores with special reference to the spores of *B. botulinus*. *J. Infectious Diseases*, **28**, 70-92 (1921).
48. Williams, C. C., Merrill, C. M., and Cameron, E. J. Apparatus for determination of spore-destruction rates. *Food Research*, **2**, 369-75 (1937).
49. Winter, A. R. Committee on Food Research, Quartermaster Food and Container Institute, Report No. 3, File # MI-5 (1946).
50. Winter, A. R. Production of pasteurized frozen egg products. *Food Technol.*, **6**, 414-15 (1952).
51. Winter, A. R., Stewart, G. F., McFarlane, V. H., and Solowey, M. Pasteurization of liquid egg products. III. Destruction of *Salmonella* in liquid whole egg. *Am. J. Pub. Health*, **36**, 451-60 (1946).

EFFECT OF SUCROSE AND ASCORBIC ACID ON QUALITY RETENTION IN FRESH AND FROZEN STRAWBERRY PUREE

D. G. GUADAGNI
Western Utilization Research Branch, Albany, Calif.*

(Manuscript received November 23, 1953)

In preparing strawberry purees for manufacture, it is important to prepare and handle the puree with maximum flavor retention. Although it is recognized that quality in fresh and frozen puree may be affected by several factors, this paper is concerned only with the effects of sugar, ascorbic acid, and delay between pureeing and freezing.

A survey of the literature indicates that most investigators consider sugar necessary in order to obtain the best flavor from frozen strawberry products *(2, 3, 4, 7, 8, 12)*. However, it is not clear whether the sugar is regarded as protecting the natural color and flavor of the berries from deteriorative changes during freezing, storage, and defrosting or whether it merely adds sweetness or accentuates the natural flavor present, or both.

Fieger *et al. (6)* reported that retention of flavor in frozen strawberries is definitely correlated with sugar penetration into the berries and that the flavor was better retained in sliced than in whole berries. Kaloyereas *(9)* found that the addition of ascorbic acid had little or no effect on quality of frozen strawberries. As early as 1920, Cruess *et al. (1)* reported that strawberries frozen without sugar made an excellent-quality ice cream. On the other hand, in 1929, Fellers and Mack *(5)* reported that strawberries frozen without sugar failed to flavor ice cream satisfactorily and that the best ice cream was obtained from strawberry packs containing 2 to 3 parts of fruit to 1 part of sucrose. Several other reports which indicated the beneficial effect of sugar on the flavor of frozen strawberries appeared during the 1930's *(2, 3, 4, 7, 8, 12)*. On the basis of these reports and our own experience with frozen strawberries and strawberry products, there is no doubt that sugar is essential for the proper balance between sweetness and sourness. It may also cause certain changes which enhance or "bring out" the natural strawberry flavor, but there does not appear to be any clear-cut evidence that sugar has any preservative effect on flavor or color or that it is capable of preventing deteriorative flavor or color changes during freezing, storage, and defrosting. For this reason, the work reported here was primarily designed to determine the effect of sugar and ascorbic acid in retaining natural color and flavor and in preventing off-flavor development in strawberry purees.

MATERIALS AND METHODS

Purees for these experiments were prepared from high-quality local fresh strawberries which were capped, washed, drained, and thoroughly mixed to insure uniformity. A screw-type machine equipped with an 0.040-inch screen was used for all purees except those prepared in a commercial plant, and those were made with a 0.062-inch screen.

*Agricultural Research Service, U. S. Department of Agriculture.

Fresh puree. The effect of holding time on the quality of fresh puree was determined at 33°F.(0.6°C.) in the absence of sugar and at 76°F.(24.4°C.) both with and without added sugar. Comparisons of stored and freshly prepared purees were made over a five-hour storage period as follows:

For each comparison a lot of uniform berries (approximately 25 pounds) was divided into two equal parts, one of which was pureed immediately and the other stored as whole berries. At hourly intervals after the first puree was made, a representative sample of the whole berries was pureed and immediately compared with portions of the stored puree. The effect of sugar was determined by adding 1 part sugar to 4 parts puree in both the stored and freshly prepared puree and comparing the two at hourly intervals. It was also determined by storing samples of the same puree with and without sugar (4 + 1 ratio). The sweetness of the unsugared sample was adjusted prior to each tasting by adding sugar in the same ratio.

Frozen purees. A large batch of puree was prepared in the laboratory and divided into 3 equal parts. One part was left untreated, another was sugared (4 + 1 ratio), and 0.2% ascorbic acid was added to the third part. Immediately after preparation, each of the purees was sealed in No. 2 enamel-lined cans and frozen in an air blast at −10°F.(−23.3°C.). All samples were in the freezer within half an hour after pureeing was begun.

After 2 months' storage at −10°F.(−23.3°C.) the samples were thawed for 2½ hours in cold running water and subjectively compared for color and flavor, both immediately after thawing and after 5 hours' storage at room temperature. All samples which were not frozen with sugar were sugared (4 + 1 ratio) just prior to each tasting.

The berries used in the preparation of commercial samples were Northwest Marshalls taken from the end of an inspection belt. These purees were divided and treated exactly as were those prepared in the laboratory. Each of the 3 purees was packed in lacquered 30-pound tins and frozen in an air blast at −40°F.(−40°C.). The frozen samples were shipped to the laboratory in Church containers packed with dry ice and stored at −10°F.(−23.3°C.) for 1 year before any comparisons were made.

Appraisal methods. All subjective comparisons were made by a panel of 10 to 15 trained judges who had previously been screened for ability to detect flavor differences in strawberries. Each comparison was repeated 2 to 4 times unless the results clearly indicated that repetition was not necessary. All fresh and frozen samples prepared in the laboratory were compared by the triangle taste test. The probability values for the results obtained with this test were taken from *Tables of the Binomial Probability Distribution (11)* and the 50-100 Binomial Tables *(10)*. The samples were randomized and served in 250-ml. beakers with approximately 100 g. of puree in each beaker. The samples prepared and stored at 33°F.(0.6°C.) were served cold and all other samples were served at room temperature under daylight lamps.

The purees which were prepared at the commercial plant were compared by 12 to 14 tasters, who used the scoring method. The comparisons were repeated 4 times and the scores for each of the purees were averaged.

RESULTS

Development of off-flavor in fresh puree. Table 1 shows that a rather rapid change occurs in quality of fresh strawberry puree stored at 33°F.(0.6°C.) and 76°F.(24.4°C.). After 2 hours' storage, significant differences in quality were beginning to develop between the stored and freshly prepared purees. The time required for the first detectable change in quality was approximately the same for samples stored at both temperatures. In view of the large differences in storage temperature, it is somewhat surprising that the first deteriorative changes should appear at the same time. However, it is interesting that the proportion of opinions of the judges who correctly identified duplicates in the samples stored at 33°F.(0.6°C.) did not increase significantly between 2 and 5 hours' storage. This indicates that even though a change in quality had definitely occurred, the rate of change during this period was very small. On the other hand, during the same storage period, there was a definite increase in the proportion of correct judgments for the samples stored at 76°F.(24.4°C.). After 3 hours at this temperature there was practically unanimous agreement that the puree had developed a marked off-flavor.

TABLE 1

Effect of length of storage on the development of off-flavor in fresh strawberry puree

Comparison	No. of judgments	No. who identified duplicates	Probability
Fresh vs.			
Stored 1 hr. at 33°F.	22	7	>.05
Stored 2 hrs. at 33°F.	35	24	<.01
Stored 3 hrs. at 33°F.	36	26	<.01
Stored 4 hrs. at 33°F.	36	24	<.01
Stored 5 hrs. at 33°F.	34	23	<.01
Stored 1 hr. at 76°F.	43	18	>.05
Stored 2 hrs. at 76°F.	28	19	<.01
Stored 3 hrs. at 76°F.	28	22	<.01
Stored 4 hrs. at 76°F.	26	24	<.01
Stored 5 hrs. at 76°F.	40	36	<.01

If the addition of sugar were to preserve natural flavor and prevent development of off-flavors in fresh strawberry puree, one would expect sugared puree to remain stable at room temperature longer than unsugared. On the contrary, Table 2 shows that sugared puree deteriorates just as rapidly as unsugared. As indicated by the proportion of correct judgments, the degree of deterioration after 3 hours' storage was more or less equivalent to that of the unsugared puree. Therefore, it is evident that quality changes occur in strawberry puree held at room temperature whether sugar is added or not. Furthermore, Table 3 indicates that there is no important difference in the degree of quality change in sugared and unsugared puree stored at 76°F.(24.4°C.).

Although a probability value of 0.03 was obtained for the samples held 5 hours, this figure is not regarded as representing an important or consistently detectable difference. After conducting hundreds of triangle tests on various strawberry samples with highly trained panels, it is our opinion that differences represented by probability values above 0.01 are of little or no importance with respect to product quality. In

TABLE 2

Effect of length of storage on the development of off-flavor in fresh sugared (4 + 1) strawberry puree held at 76°F.(24.4°C.)

Comparison	No. of judgments	No. who identified duplicates	Probability
Fresh[1] vs.			
Stored 1 hr.	27	20	<.01
Stored 2 hrs.	26	20	<.01
Stored 3 hrs.	28	26	<.01
Stored 4 hrs.	28	26	<.01
Stored 5 hrs.	13	13	<.01

[1]One part sugar added to 4 parts of freshly prepared puree just before tasting.

TABLE 3

Comparison of fresh sugared (4 + 1) and unsugared strawberry puree held at 76°F.(24.4°C.) for varying times[1]

Storage	No. of judgments	No. who identified duplicates	Probability
1 hr.	24	11	>.05
3 hrs.	20	10	>.05
5 hrs.	22	12	.03

[1]One part sugar added to 4 parts of unsugared puree just prior to tasting.

view of this experience, it is doubtful that sugar exerted any protective effect against the deteriorative changes that occurred in fresh strawberry puree.

Effect of sugar and ascorbic acid in frozen puree prepared in the laboratory. Data in Table 4 show that there was no significant difference between purees frozen with and without sugar after 2 months' storage at −10°F.(−23.3°C.) and 2.5 hours of defrosting. Comparison of the frozen purees was repeated on 4 separate days, and although there was considerable variation in the number of correct judgments, these were not sufficient to indicate a detectable difference. When the same purees were allowed to stand at room temperature 5 hours after defrosting, a probability value of 0.02 was obtained. As pointed out above, this result indicates a difference between the 2 samples but is not considered an important or consistently detectable difference. Furthermore, since sugar has been shown to be ineffective against quality deterioration in fresh puree, it is difficult to be certain that this result on frozen thawed puree was due to any protective effect of sugar.

Data obtained by comparing frozen purees prepared with 0.2% and without added ascorbic acid show that the addition of ascorbic acid did not cause any significant difference in the quality of the frozen puree. This was true of the samples held at room temperature 5 hours after defrosting, as well as those tasted immediately after thawing.

Comparison of the frozen samples prepared with sugar (4 + 1) and ascorbic acid (0.2%) indicated that there was no important difference between these samples either immediately or 5 hours after defrosting. Since the ascorbic acid samples were frozen and stored without added sugar, the results of this comparison contribute additional evidence that sugar is not necessary for quality retention during frozen storage and extended thawing.

Effect of sugar and ascorbic acid in frozen puree prepared in a commercial plant. Although statistical calculations were not made on the data shown in Table 5, the

TABLE 4

Comparison of sugared, unsugared, and ascorbic acid-treated frozen strawberry puree prepared in the laboratory

Comparison[1]	No. of judgments	No. who identified duplicates	Probability
S_1 vs. S_2 immediately after thawing	53	19	>.05
S_1 vs. S_2 stored 5 hrs. at 76°F. after defrosting	38	19	.02
S_1 vs. S_2 immediately after thawing	50	13	>.05
S_1 vs. S_2 stored 5 hrs. at 76°F. after defrosting	50	19	>.05
S_2 vs. S_3 immediately after thawing	52	23	>.05
S_2 vs. S_3 stored 5 hrs. at 76°F. after defrosting	47	19	>.05

[1] S_1 = unsugared. S_2 = 0.2% ascorbic acid added prior to freezing, no sugar added. S_3 = 1 part sugar added to 4 parts puree prior to freezing, 1 part sugar added to 4 parts of S_1 and S_2 just prior to tasting.

TABLE 5

Comparison of sugared, unsugared, and ascorbic acid-treated frozen strawberry puree prepared in a commercial plant

Replication	Samples compared immediately after defrosting[1]			Samples compared after defrosting and 6 hours' storage at 40°F.(4.4°C.)		
	4 + 1 sugar	0.2% ascorbic acid	Untreated	4 + 1 sugar	0.2% ascorbic acid	Untreated
1	3.6	4.4	4.3	3.8	4.2	4.0
2	3.9	4.3	4.3	4.1	4.4	3.9
3	4.0	4.5	4.4	4.0	4.2	4.1
4	3.5	4.3	4.3	3.9	4.3	4.0

[1] Average flavor scores: 5 indicates that the sample was very desirable, and scores of 4 down to 1 indicate decreasing desirability.

average scores for the 4 replications indicated that packing and storing with sugar or ascorbic acid had no advantage over the untreated puree. After one year of storage at −10°F.(−23.3°C.), the flavor of the untreated puree was as good as the sugar- and ascorbic-acid treated samples. As a matter of fact, the flavor scores for the sugared puree, for some unexplainable reason, were somewhat lower than those of the unsugared or ascorbic-acid treated. When the thawed purees were allowed to stand at 40°F.(4.4°C.) for 6 hours, the scores of the 3 samples were even closer together than the scores obtained immediately after thawing. Since practically all the phases of fruit preparation such as harvesting, washing, sorting, pureeing, packaging, and freezing were done by commercial methods, these results indicate what might be expected in actual commercial preparation of strawberry purees.

DISCUSSION

At this point it must be emphasized that the results obtained on puree may not apply to whole or sliced berries. Packing these products with sugar may offer advantages, especially with regard to texture.

Probably the main reason for the ineffectiveness of sugar in these experiments was the fact that all comparisons were made at or near the same level of sweetness, and therefore flavor judgments were not influenced by this factor. Unless this precaution were taken, sugared samples would be easily recognized and preferred even when no real difference in natural strawberry flavor existed. Results obtained with this procedure showed that strawberry puree can be frozen, stored, and defrosted in the absence of sugar without apparent sacrifice of quality.

This may have commercial importance in that refrigeration and shipping costs would be reduced if no sugar were added at time of packing. Since puree is almost entirely used for remanufacture, the overall amount of sugar utilized in strawberry products would not be reduced by packing without sugar. These products are usually standardized with respect to sugar content, and therefore the amount added during remanufacturing would merely be increased if the fruit were received without added sugar.

SUMMARY

Experiments designed to show the effect of sucrose and ascorbic acid on the quality of fresh and frozen strawberry puree are described. The results show that deteriorative changes in the quality of fresh puree occur within 2 to 3 hours at room temperature and that sucrose in a (4 + 1) ratio neither prevents nor minimizes these changes. In frozen purees, the addition of sucrose or ascorbic acid offered no advantage with respect to quality retention during freezing, storage, and defrosting.

Acknowledgment

The assistance of Virginia Cushman in preparing the samples for organoleptic appraisal is gratefully acknowledged.

LITERATURE CITED

1. Cruess, W. V., Overholser, E. L., and Bjarnson, S. A. Storage of perishable fruits at freezing temperatures. Calif. Agr. Expt. Sta. *Bul. No. 324* (Aug., 1920).
2. Diehl, H. C. The cold pack method of preserving berries in the Pacific Northwest. *Glass Packer*, **2**, 115 (1929).
3. Diehl, H. C. Preserving fruits and vegetables by freezing. *The Glass Container*, **9** (4), 8 (Feb., 1930).

4. DIEHL, H. C. Freezing of fruits and vegetables requires more research. *Food Inds.*, 2 (4), 162 (1930).
5. FELLERS, C. R., AND MACK, M. J. Utilization of cold packed fruits in frozen dairy products. III. *Fruit Prod. J.*, **9**, 108 (1929).
6. FIEGER, E. A., DUBOIS, C. W., AND KALOYEREAS, S. Freezing experiments on strawberries. *Fruit Prod. J.*, **25**, 297 (1946).
7. IRELAND, ROBERT. Cold-packed barreled fruit in 1930. *Food Inds.*, **3**, 214 (1931).
8. JOSLYN, M. A. Why freeze fruit in sirup? *Food Inds.*, **2**, 350 (1930).
9. KALOYEREAS, S. Strawberry studies, a new method for determining firmness. *Fruit Prod. J.*, **27**, 6 (Sept., 1947).
10. ROMIG, HARRY G. 50-100 Binomial Tables. 1953, John Wiley and Sons, Inc., New York, N. Y.
11. U. S. National Bureau of Standards. *Tables of the Binomial Probability Distribution. Applied Mathematics. Series No. 6* (1950).
12. WIEGAND, E. H. The "frozen-pack" method of preserving berries. Oreg. Agr. Expt. Sta. *Bul. No. 278* (May, 1931).

THE DIETHYLSTILBESTROL CONTENT OF TISSUES OF TREATED STEERS, LAMBS, AND POULTRY

C. E. SWIFT

Agricultural Research Service, U. S. Department of Agriculture, Agricultural Research Center, Beltsville, Maryland

(Manuscript received November 28, 1953)

Practical use of diethylstilbestrol treatments in animal and poultry husbandry depends on there being no objectionable residual quantities of the chemical in edible tissues, for otherwise other considerations will be meaningless. Several reports of analyses of tissues of poultry have been made *(1, 3, 4)*. Recently the subject has been reviewed *(5)* and the need emphasized for improved methods of analysis and for systematic analysis of tissues of treated animals and poultry. During an investigation of the residual diethylstilbestrol in the tissues of treated steers, lambs, and poultry, the chemical method described here for the concentration and determination of free diethylstilbestrol was developed and applied. While the work was principally concerned with the determination of free diethylstilbestrol, in the analysis of poultry tissues both the combined and the free forms were determined, yielding results which are reported and interpreted with necessary qualifications. Although no evidence of the presence of the synthetic estrogen was found in analyses of extracts of tissues of treated lambs or a steer, evidence was found of small amounts in extracts of the liver and skin, but not the muscle tissue, of treated poultry.

METHODS, PROCEDURE, AND RESULTS

Reagents and materials. Petroleum ether (b.p. 60-70°C.) was thrice extracted with 0.1 volume of concentrated sulfuric acid, then successively with 0.1 volumes of water, 1 N sodium hydroxide and again with water, after which the solvent was distilled.

Acetone, A.C.S., was distilled through a short column discarding a residue of approximately 10%.

Ethyl ether, anhydrous, A.C.S., was refluxed with and distilled from an equal volume of a solution containing 150 g. of ferrous sulfate, and 15 ml. of concentrated sulfuric acid in 275 ml. of water.

Absolute ethanol was distilled discarding a small colored residue.

Petroleum ether (b.p. 37-40°C.) used in paper chromatography was obtained by fractionally distilling solvent having b.p. 30-60°C.

Buffer solution. The solution was prepared by mixing 800 ml. of glacial acetic acid, 150 ml. of 10% potassium hydroxide solution (by weight) and 50 ml. of water.

Alcoholic ammonium hydroxide solution. The solution was freshly prepared by mixing 25 ml. of absolute ethanol with 22.5 ml. of concentrated ammonium hydroxide and 2.5 ml. of water.

Saturated aqueous sodium nitrate solution, freshly prepared.

Concentrated sulfuric acid.

Concentrated hydrochloric acid.

Dilute hydrochloric acid solution, concentrated hydrochloric acid diluted 1:10.

Sodium hydroxide solution, approximately 1 N.

Sodium carbonate solution, 10%, by weight, freshly prepared.

Sodium bicarbonate solution, 9% by weight.

Phosphoric acid solution (9 N).

Whatman No. 1 filter paper.

Extraction and concentration. Samples consisting only of fatty tissue were ground through a meat grinder. To 100 g. of ground tissue 150 ml. of petroleum ether was added and this mixture was stirred and warmed on a steam bath until the lipids dissolved and separated from cellular tissues. The warm solution was filtered and extracted with three 50-ml. portions of 1 N sodium hydroxide. If an emulsion interfered with separations, the mixture was separated by centrifugation. The alkaline extracts were combined and filtered and the volume of the filtrate was measured to allow correction for unrecovered solution. The solution was extracted with 50 ml. of petroleum ether which was discarded. Concentrated hydrochloric acid was added to the alkaline solution to adjust it to pH 3. The acidified solution was extracted with three 50-ml. portions of ethyl ether. The ethereal extracts were combined and extracted successively with three 15-ml. portions of 10% sodium carbonate, 15 ml. of dilute hydrochloric acid and 15 ml. of water, after which the solvent was evaporated on a steam bath under an atmosphere of nitrogen and a residue was recovered. If more than a barely visible film of oily material appeared, the residue was again dissolved in 150 ml. of ethyl ether and extracted with three 50-ml. portions of 1 N sodium hydroxide. The combined alkaline extracts were acidified with concentrated hydrochloric acid to *ca.* pH 3 and the acidified solution extracted with three 50-ml. portions of ethyl ether. The ethereal solvent was evaporated as before and the residue recovered.

Muscle and liver tissues were also ground through a meat grinder and 100-g. samples, otherwise whatever lesser amounts were available such as 25-35 g. chicken livers (Group I), 90-100 g. chicken livers (Group II) or 90 g. chicken skins, were extracted with acetone as has previously been described *(3)*. The acetone extracts were combined and concentrated under vacuum to 140 ml.; 90 ml. of water was then added and the mixture was extracted with three 85-ml. portions of a mixture of 35 parts of ethyl ether and 65 parts petroleum ether (by volume). The 3 ethyl ether-petroleum ether extracts were combined and extracted first with four 75-ml. portions of 9% sodium bicarbonate to remove conjugated diethylstilbestrol and then with three 50-ml. portions of 1 N sodium hydroxide. The sodium hydroxide extracts were combined, filtered and the volume of the filtrate was determined. This filtrate was then treated as were corresponding filtrates in the above-described analyses of fatty tissue.

Hydrolysis of conjugated fractions. The four 75-ml. extracts made with 9% sodium bicarbonate were combined; 9 N phosphoric acid was added to adjust the solution to a pH of from 2 to 3 and the acidified solution was extracted with three 50-ml. portions of ethyl ether. The ethereal extracts were combined and the solvent was evaporated. The residue which was obtained was heated 90 minutes at 180°C. in a sodium hydroxide-phosphoric acid buffer at pH 3.45, this method having previously been effective in the hydrolysis of diethylstilbestrol glucoronide *(6)*. In the present work the buffered solution was sealed in a Carius tube during hydrolysis. After the hydrolysis, 1 N sodium hydroxide was added to adjust the solution to *ca.* pH 12 and the solution was extracted with 50 ml. of petroleum ether. The petroleum ether extract was discarded; 9 N phosphoric acid was added to the alkaline solution to adjust it to a pH of from 2 to 3 and the acidified solution was extracted with three 50-ml. portions of ethyl ether. The ethereal extracts were combined and extracted successively with three 15-ml. portions of 10% sodium carbonate, 15 ml. of dilute hydrochloric acid and 15 ml. of water; the solvent was evaporated on a steam bath under an atmosphere of nitrogen and a residue was obtained.

Chromatography. Each residue obtained by any of the three above-described procedures was fractionated as follows: The residue was dissolved in small amounts of ethyl ether and ethanol and transferred, using a capillary pipette, to a strip (2.5 × 25 cm.) of Whatman No. 1 filter paper. The material was transferred so as to form a band approximately 2 cm. in depth extending across the strip approximately 5 cm. from one end. The strip was placed in a test tube (2.8 cm. width, 28 cm. length) containing 2-3 ml. of petroleum ether (b.p. 37-40°C.) and the solvent was allowed to ascend 20 to 25 cm. The strip was then placed in a similar test tube containing 2-3 ml. of a mixture of equal portions of petroleum ether (37-40°C.) and benzene. The solvent was again allowed to ascend 20-25 cm. The paper was withdrawn, air-dried, and cut to remove the uppermost bands, usually an orange-colored and an oily band. The shortened strip of paper, now approximately 20 cm. in length, was placed in a test tube containing 2-3 ml. of benzene and the solvent was allowed to ascend to, or nearly

to, the top of the paper. The strip was then cut at the bottom to remove the section containing colored bands which had not moved during the various elutions. Test tubes were closed with stoppers during the chromatographic procedures. The remaining (middle) portion of the strip was suspended under a reflux condenser and extracted for 90 minutes with refluxing ethanol. The ethanol extract (15-20 ml.) was transferred to a 50-ml. beaker and the solvent evaporated on a steam bath under an atmosphere of nitrogen.

Determination of diethylstilbestrol. The method described by Gottlieb *(3)*, modified principally by reducing the proportions of reactants, was employed as follows: To the residue 1 ml. of acetic acid buffer solution was added from a pipette; a drop of concentrated sulfuric acid followed by a drop of freshly prepared saturated sodium nitrite was added. The mixture was occasionally swirled and allowed to stand for 30 minutes. The beaker containing the reactants was then cooled in an ice bath and 4 ml. of alcoholic ammonium hydroxide solution were added from a pipette. The resulting solution was allowed to stand at room temperature for 30 minutes after which it was filtered and its optical density at 440 mμ was determined in a Beckmann DU spectrophotometer with the blue-sensitive phototube in position. A blank consisting of alcoholic ammonium hydroxide solution was used. From the optical density and reference to a standard curve prepared by determining 0-100 γ of diethylstilbestrol under the same conditions, the quantity of chromogenic substance expressed as diethylstilbestrol in the residue was determined; the content of samples was then calculated, using the equation

$$\text{Total chromogenic substance expressed as diethylstilbestrol, } \gamma/100 \text{ g.} = \frac{100}{\text{wt. of sample, g.}} \times \frac{150}{\text{ml. 1 N alkali in filtrate}} \times \text{chromogenic substances in residue, } \gamma$$

The correction for loss of alkaline solution was omitted in calculating the quantities obtained from the conjugated fraction.

Treatments of animals and poultry with diethylstilbestrol. A milking Shorthorn steer which received an initial implant of 120 mg. of diethylstilbestrol and an additional implant of 120 mg. after 30 days, was slaughtered to furnish tissues for analyses. The steer had an average daily gain of 3.2 lbs. on a ration of corn, cottonseed meal, and grass hay. Slaughter followed 109 days after the first treatment. The carcass was quartered and stored at −18°C., analyses being made after approximately 40 days of such storage.

The tissues of lambs which were analyzed were obtained from 3 pairs of wether lambs which were initially treated with 15-, 30-, and 60-mg. implants. Approximately 30 days before slaughter the dosages were repeated. The lambs were slaughtered approximately 60 to 90 days after their first treatment and their carcasses were stored at −18°C. Samples were withdrawn for analyses over a period of approximately 6 weeks.

A large group (Group I) of 6-week old cockerels was divided into 5 groups, each consisting of four or more birds, providing a control group and groups which were treated with diethylstilbestrol implanted subcutaneously in their necks at 15-, 30-, 60-, and 120-mg. levels. After 35 days they were killed and their carcasses separated into liver, skin, leg and back muscles, and breast muscle. These portions of the carcasses were sealed in jars and frozen until withdrawn for analysis over a period of 16 weeks.

One-half of a 6-week old group (Group II) of cockerels was treated with 15-mg. pellets. After 4 weeks both control and treated birds were killed and their livers removed. The livers were frozen and stored for less than one week during which period samples were withdrawn and analyzed.

Analyses. Quantities ranging from 22 to 105 γ of diethylstilbestrol were added to 100 g. of several types of tissues and analyses were performed to determine the amounts recoverable. The known quantities were dissolved in ethyl ether and added to the ground tissues after which the solvent was evaporated. The amounts added and the chromogenic substances recovered are shown in Table 1. Table 1 also shows the results of determinations on tissues of untreated steers and lambs. Results of analyses of tissues of treated lambs and a steer are shown in Table 2 and those obtained on tissues of treated and untreated poultry in Table 3. In Table 2 averages of duplicate

determinations are shown; in Table 3 averages as well as individual determinations are given. Samples consisted of single livers in analyses of livers of birds in Group I, while each sample consisted of one-third of the livers of 9 birds in the analyses of tissues of birds in Group II and the analyses were performed in triplicate.

TABLE 1

Recovery of known amounts of diethylstilbestrol added to 100 g. tissue

Tissue	Chromogenic substances[1] in original tissue[2]	Diethylstilbestrol added	Diethylstilbestrol and other chromogenic substances			
			Calculated total	Recovered[3]		
	γ	γ	γ	γ	%	
Beef cod fat	4.0	29.5	33.5	32.0	95.4	89.1 av.
Beef cod fat	4.0	55.5	59.5	48.0	80.7	
Lamb caul fat	11.0	105.0	116.0	106.0	91.3	
Beef muscle	10.0	22.0	32.0	19.0	60.0	64.2 av.
Lamb muscle	11.0	26.0	38.0	26.0	68.4	
Beef liver	9.0	32.5	41.5	29.0	69.9	75.3 av.
Beef liver	4.0	29.5	33.5	27.0	80.6	

[1]Expressed as diethylstilbestrol.

[2]Tissues analyzed were from untreated animals; also, in livers of untreated animals not included above, analyses indicated 14 and 10 γ in beef and lamb livers, respectively.

[3]$\frac{\text{Diethylstilbestrol and other chromogenic substances recovered, } \gamma}{\text{Chromogenic substances in original tissues, } \gamma + \text{Diethylstilbestrol added, } \gamma} \times 100 = \text{Diethylstilbestrol and other chromogenic substances recovered, per cent}$

DISCUSSION

Table 1 shows data obtained in determinations of known amounts of diethylstilbestrol added to tissues. Recovery of chromogenic substances, expressed as diethylstilbestrol, ranged from 60.0 to 95.4%, averaging 64.2, 75.3, and 89.1% in determinations of diethylstilbestrol added to muscle, liver, and fatty tissues, respectively. It can be assumed that the 10.9% (average) loss of diethylstilbestrol in analyzing fatty tissues represents the sum of losses occurring during the isolation of the synthetic estrogen; however, a large part of the losses of diethylstilbestrol added to muscle or liver is assumed to be attributable to the inability of acetone to completely extract diethylstilbestrol bound to protein. Extraction of tissues mixed with equal amounts of anhydrous sodium sulfate with ethyl ether in a Soxhlet extractor gave approximately the same results.

The present data were not adjusted to compensate for incompleteness of recovery since such adjustments would not affect the range of concentrations of the chemical found in the tissues to such an extent as to alter the principal conclusions of the work.

Small amounts of chromogenic substances were obtained from tissues of untreated animals and poultry under the conditions of the determination, despite efforts to develop means of eliminating compounds other than diethylstilbestrol. To establish a basis for determining and compensating for the effect of these interfering substances, determinations on tissues of untreated as well as treated animals and poultry were undertaken. The identity of the interfering compounds is not known. It has been shown that the nitroso compounds formed with estrone and α-estradiol have a

comparatively low absorption and do not appreciably interfere with the determination of diethylstilbestrol as a nitroso compound *(2)*. The interference existed to a decreasing extent from 400 to 500 mμ, exhibiting no maximum or minimum. In determinations on tissues to which approximately 50 γ or more of diethylstilbestrol were added, the maximum at 440 mμ of the nitroso compounds formed from diethylstilbestrol was discernible in absorption curves plotted over the range 400 to 500 mμ, but not when smaller amounts were added.

TABLE 2

Content of chromogenic substances, expressed as diethylstilbestrol, of 100 g. of tissues of treated lambs and a steer.

Animals and treatment	Tissue	Chromogenic substances[1]
		γ
Steer, total of 240 mg. implanted	Subcutaneous fat inside flank	6
	Ruffle fat	6
	Caul fat	9
	Cod fat	5
	Muscle from short ribs	8
	Liver	7
Lamb 601, total of 30 mg. implanted	Caul fat	8
Lamb 728, total of 60 mg. implanted	Caul fat	9
	Liver	15
Lamb 828, total of 120 mg. implanted	Caul fat	11
	Subcutaneous fat from leg	9
	Muscle of leg	11
	Liver	12

[1]Expressed as diethylstilbestrol.

Table 2 shows the amounts of chromogenic substances, expressed as diethylstilbestrol, found in the extracts of the tissues of treated lambs and a steer. The apparent diethylstilbestrol content of these tissues was in the range of that of tissues from untreated animals (Table 1), indicating the existence of no appreciable free diethylstilbestrol in them.

Table 3 shows the amounts of both free and combined chromogens and the calculated apparent diethylstilbestrol content of extract of tissues of treated poultry. The data indicate that small residues of the chemical are to be expected in the liver and skin, but not the muscles of treated poultry. Other workers have reported *(4)* that livers of chickens treated with 15 mg. of diethylstilbestrol contained less than 125 γ, total, and perhaps much less. Using the average apparent content of diethylstilbestrol of tissues of birds in Group I as a basis for calculation, and assuming the liver to weigh 33⅓ g. and the skin 100 g., a chicken treated with the conventional 15 mg. pellet contained 37 $\gamma \left(\frac{32}{3} + 26\right)$. The apparent diethylstilbestrol content of livers of birds in Group II was small and is not considered to be a positive indication of the presence of diethylstilbestrol. As indicated by the results of analyses of individual livers of birds in Group I, the

TABLE 3

Apparent diethylstilbestrol content (γ per 100 g.) of tissues of treated poultry

Group	Treatment	Total chromogenic substances[1]				Apparent content of diethylstilbestrol[2]			
		Liver	Skin	Breast muscle	Back and leg muscle	Liver	Skin	Breast muscle	Back and leg muscle
I	Control								
	Free	18 (13, 22)	9 (9, 10, 9)	17 (25, 9)	9 (4, 14)	....	....	....	....
	Combined[3]	14 (12, 15)	4 (4, 4)	10 (12, 7)	7 (7, 7)	....	....	....	....
	Total	32	13	27	16	....	....	....	....
	15 mg.								
	Free	26 (20, 32)	29 (23, 41, 23)	....	....	8	20	....	....
	Combined	38 (29, 47)	11 (6, 12, 14)	....	....	24	7	....	....
	Total	64	40	....	....	32	27	....	....
	30 mg.								
	Free	39 (12, 78, 32, 34)	24 (31, 24, 17)	....	....	21	15	....	....
	Combined	16 (11, 22, 12, 20)	11 (11)	....	....	2	7	....	....
	Total	55	35	....	....	23	22	....	....
	60 mg.								
	Free	34 (26, 16, 51, 40)	25 (8, 54, 12)	....	....	16	16	....	....
	Combined	42 (29, 54)	9 (7, 4, 9, 14)	....	....	28	5	....	....
	Total	76	34	....	....	44	21	....	....
	120 mg.								
	Free	60 (59, 83, 37)	27 (25, 35, 22)	16 (10, 21)	11 (9, 12)	42	18	0	2
	Combined	40 (38, 42)	11 (12, 10)	19 (17, 20)	10 (9, 10)	26	7	9	3
	Total	100	38	35	21	68	25	9	5
II	Control								
	Free	16 (11, 16, 21)	....	....	....	....	....	....	....
	Combined	6 (4, 8, 7)	....	....	....	....	....	....	....
	Total	22	....	....	....	....	....	....	....
	15 mg.								
	Free	18 (23, 16, 16)	....	....	....	2	....	....	....
	Combined	8 (7, 9, 9)	....	....	....	2	....	....	....
	Total	26	....	....	....	4	....	....	....

[1] Averages are given, also enclosed in parentheses are the results of individual determinations.
[2] Calculated by determining the difference between values obtained on treated and untreated tissues.
[3] Determined on purified hydrolysates of conjugated fractions.

content of diethylstilbestrol in the livers of treated birds was highly variable while, on the other hand, the amounts of chromogenic substances in the livers of control birds in both Groups I and II were reasonably constant. Variations of amounts of synthetic estrogen retained in the tissues are of course to be expected, as are variations in the growth responses of treated birds. The averages of the diethylstilbestrol content of the group treated with increasing amounts of the chemical appear to reflect, although imperfectly because of the influence of variation within groups, an increase in content of diethylstilbestrol with increase in the amounts used in the treatments, the greatest concentration being 68 γ per 100 g. in livers of birds treated with 120 mg., or eight times the normal dosage.

In none of the data, therefore, was there an indication that appreciable diethylstilbestrol residues are to be expected in tissues of conventionally treated animals or poultry, excluding the immediate areas in which the chemical was injected. Recognizing that diethylstilbestrol may be bound so as not to be extracted and determined by ordinary chemical methods, rigorous proof is lacking that accurate estimates have yet been made; hence it is suggested that further work on the problem, perhaps based on the use and analysis of radioactive diethylstilbestrol, be undertaken.

SUMMARY

A method has been developed for the determination of diethylstilbestrol in the fatty, liver, and muscle tissues of steers, lambs, and chickens.

In analyses of extracts of tissues of lambs and a steer which had been treated with diethylstilbestrol no evidence of the presence of the synthetic estrogen was found.

In analyses of extracts of tissues of chickens treated with from one to eight 15-mg. pellets of diethylstilbestrol, data were obtained indicating not in muscle tissue. In the extracts of the liver and skin of a chicken receiving the conventional one-pellet treatment the diethylstilbestrol content was calculated, on the basis of analyses on one group of chickens, to be only 37 γ, or *ca.* 0.25% of the amount implanted.

Further work on determining diethylstilbestrol in its bound forms is recommended to exclude the possibility of there being as yet undetected residues of combined chemical in the tissue.

Acknowledgment

The author wishes to acknowledge the assistance of members of the Animal Husbandry Division, as follows: E. H. McNally in supplying poultry tissues; Ivan L. Lindahl in supplying diethylstilbestrol-treated lambs and steers, and Rex Ellis in assisting with analyses.

LITERATURE CITED

1. Bird, S., Pugsley, L. I., and Klotz, M. D. The quantitative recovery of synthetic estrogens from the tissues of birds *(callus domesticus)*; the response of the birds' testes, comb and epidermis to estrogen and of humans to ingestion of tissues from treated birds. *Endocrinology*, **41**, 282 (1947).

2. Gottlieb, S. J. Chemical determinations of synthetic estrogens. I. Basic data on the nitrosophenol reaction. *J. Am. Pharm. Assoc., Sci. Ed.*, **26**, 379 (1947). II. Application to pharmaceutical preparations of diethylstilbestrol. *J. Am. Pharm. Assoc., Sci. Ed.*, **27**, 147 (1948).

3. GOWE, R. S. Residual estrogens in the tissues of fowl treated with dienestrol diacetate. *Poultry Sci.*, **28**, 666 (1949).

4. JONES, O., AND DEATHERAGE, F. E. The diethylstilbestrol content of the meat from chickens treated with this estrogenic substance. *Food Research*, **18**, 30 (1953).

5. SYKES, J. F., *et al.* Hormonal relationships and applications in the production of meats, milk and eggs. Committee on Animal Nutrition. Subcommittee on Hormones, Agricultural Board, National Academy of Science, National Research Council, March 1953.

6. TEAGUE, R. S., AND BROWN, A. E. Determination of diethylstilbestrol and its glucoronide in urine. *J. Biol. Chem.*, **189**, 343 (1951).

EFFECT OF AGING AND COOKING ON THE DISTRIBUTION OF CERTAIN AMINO ACIDS AND NITROGEN IN BEEF MUSCLE[a]

IRENE D. GINGER, JAMES P. WACHTER, D. M. DOTY, AND B. S. SCHWEIGERT

Divisions of Biochemistry and Nutrition and Physical and Analytical Chemistry, American Meat Institute Foundation and Department of Biochemistry, University of Chicago, Chicago, Illinois

AND

F. J. BEARD, J. C. PIERCE, AND O. G. HANKINS[b]

U. S. Department of Agriculture

(Manuscript received December 5, 1953)

In recent years much work has been done to determine the amino acid composition of fresh, cooked and processed meats, and meat products. The distribution of microbiologically available amino acids and the total amino acid content of extracts of meat and drippings from cooked meat has had little or no attention. The amounts of leucine, valine and isoleucine in water extracts of veal and lamb muscle have been studied [Schweigert, *et al. (4)*], and the free amino acid content of tungstic acid extracts of rat muscle has been investigated [Thompson, *et al. (7)*].

Several investigators have studied various gross nitrogenous fractions from beef. Hiner, Gaddis and Hankins *(1)* recently reported the amount of soluble, non-protein and amino acid nitrogen in raw, frozen and frozen stored beef. Husaini, *et al. (2)* determined the non-protein nitrogen, trichloroacetic acid soluble nitrogen and water soluble, heat coagulable nitrogen in the short loin of beef from 20 carcasses.

In the present study, the amino acid content was determined in the fresh and aged raw and cooked meat, in extracts prepared from these samples and in the drippings from the cooked meat by the use of microbiological methods before and after acid hydrolysis of the samples. The various other nitrogenous fractions were determined in the fresh and aged raw and cooked meat and in the drippings. Information on the relative amounts of "free" and "bound" amino acids in the extracts and the drippings has indicated the nature of proteolytic changes that occurred during aging and cooking in terms of specific amino acids. The losses of arginine, leucine, and tyrosine in the drippings during cooking were small (less than 3% of the total in the cut) and increased slightly after aging of the meat. The amount of histidine in the drippings was greater (7% of the total in the cut), and further studies were conducted on the nature of the histidine peptides present in the drippings and NPN fractions of beef muscle.

[a]Journal Paper No. 83, American Meat Institute Foundation. This work was conducted at the American Meat Institute Foundation, and was supported in part by Research and Marketing Funds under contract with the United States Department of Agriculture.

[b]Deceased.

EXPERIMENTAL

Paired beef rib cuts were obtained (2 days post mortem) and a steak from each pair was used for analysis of the uncooked meat, while the other was broiled at 400°F. to an internal temperature of 150°F. before analysis. Adjacent paired steaks were treated in a similar manner after aging in the cut for two weeks at 35°F.

The edible portion of each steak was removed from the bone, ground 3 times and thoroughly mixed. A portion of the sample was removed for chemical analyses and a portion retained for the determination of the content and distribution of certain amino acids. The drippings from each cooked steak were collected, suspended in water, the fat extracted with diethyl ether, the fat-free water suspension filtered, and the filtrate used for analysis.

The "non-protein nitrogen" (NPN) fraction was obtained by extraction of the sample in the Waring blender with phosphate buffer at pH 6.5 followed by the addition of trichloroacetic acid (4% final concentration) to the extract. Non-protein nitrogen was determined by the Kjeldahl method on a suitable aliquot of the filtrate after trichloroacetic acid precipitation. The nitrogen in the soluble protein fraction was determined by the Kjeldahl method on the trichloroacetic acid precipitate. Amino nitrogen was determined in the NPN and dripping filtrates with the Van Slyke gasometric apparatus, using a 5-minute reaction period with a shaking speed of 80 r.p.m.

The amino acid content of raw and cooked meat samples was determined after acid hydrolysis (5 g. meat in 50 ml. 3*N* HCl for 16 hrs. at 15 p.s.i.). The amino acids determined were leucine, tyrosine, histidine, arginine and for certain samples lysine and glutamic acid. The drippings from the cooked samples and the NPN extracts of all samples were analyzed for amino acid content before and after hydrolysis. For the hydrolysis a suitable aliquot was autoclaved with HCl (final concentration 1*N*) for 16 hours at 15 p.s.i. Suitable dilutions were made of each sample after neutralization, and the amino acids were determined microbiologically with the use of the detailed procedure and media described by Schweigert, *et al.* (5).

Leucine and tyrosine were determined with *Lactobacillus arabinosus* 17-5 as test organism, arginine with *Streptococcus faecalis* R, and histidine with *Streptococcus faecalis R* and *Leuconostoc mesenteroides* P-60 as test organisms.

Some inhibition of growth was observed when the unhydrolyzed NPN fraction was analyzed for some of the amino acids. Experiments with the use of known concentrations of amino acids and a salt concentration higher than that in the unhydrolyzed NPN fractions showed that the salt concentration of these fractions was not the inhibitory factor. When the unhydrolyzed NPN fraction was Seitz-filtered and added aseptically in the microbiological assay there was no apparent inhibition. This method was used in determining the amino acids in the unhydrolyzed NPN fractions.

The concentrations of tyrosine and leucine in the hydrolyzed NPN fraction were, in some cases, too low to give reliable results. In these cases the values reported are based on recovery experiments.

The values for the amino acid content of the meat, drippings, and most NPN fractions are averages of two or more assays. The variation from the mean for 44 duplicate or triplicate analyses on the drippings, for example, averaged ± 9.5% (range 0.6 to 25.5%).

RESULTS AND DISCUSSION

Results of the determination of moisture, ether extract and the various nitrogen fractions in the samples are presented in Table 1. Only a very small percentage of the nitrogen present in the raw meat was lost in the drippings regardless of meat grade or extent of aging. The nitrogen in the drippings was essentially all non-protein nitrogen with a fairly high proportion (14-25%) determined as free amino nitrogen.

The NPN content of the cooked steaks was less than that of the raw meat; however, the loss could largely be accounted for by the nitrogen in the drippings. No consistent change in the amino acid content of the NPN fractions occurred during cooking (Tables 2 and 3). Cooking caused a very marked decrease (4- to 30-fold) in the soluble protein nitrogen of the steaks.

TABLE 1

The moisture, ether extract and protein content and nitrogen distribution in beef rib steaks

Grade and aging	Sample description	Moisture[1] %	Ether extract[1] %	Protein[1] (N×625) %	NPN		Sol. Protein N		Amino N	
					g.[2]	% of total N[2]	g.[2]	% of total N[2]	g.[2]	% of total N[2]
Unaged	Raw	47.8	36.8	14.7	1.37	7.6	3.14	17.5	0.19	1.1
Prime	Cooked	45.4	29.5	23.4	1.24	6.9	0.73	4.1	0.19	1.1
Rib steak	Drippings	.....	.....	.....	0.38	2.1	0	0	0.08	0.4
Aged	Raw	48.4	36.6	15.0	1.40	7.1	1.32	6.7	0.25	1.3
Prime	Cooked	42.6	35.3	21.7	0.61	3.1	0.12	0.6	0.23	1.2
Rib steak	Drippings	.....	.....	.....	0.35	1.8	0	0	0.08	0.4
Unaged	Raw	53.7	29.4	17.5	0.97	7.9	2.14	17.3	0.09	0.8
Good grade	Cooked	45.1	29.6	25.1	0.68	5.5	0.07	0.6	0.09	0.8
Rib steak	Drippings	.....	.....	.....	0.28	2.3	0	0	0.04	0.3
Aged	Raw	48.3	35.1	15.6	1.12	10.4	2.83	26.4	0.12	1.1
Good grade	Cooked	42.8	32.6	23.6	0.84	7.9	0.39	3.6	0.11	1.0
Rib steak	Drippings	.....	.....	.....	0.23	2.2	0	0	0.04	0.3

[1] These analyses were made by the Service Laboratory, American Meat Institute Foundation.
[2] All values expressed as total amount in the edible portion of the steak and as per cent of the total nitrogen present in the raw steak.

Aging increased the amino nitrogen content of the NPN fraction of both raw and cooked meat. The amount in the cooked steak plus drippings was always greater than that of the raw meat, which shows that some proteolysis occurred during cooking. The distribution of certain amino acids in the unhydrolyzed extracts followed that of amino nitrogen for some of the samples. Extensive studies on the effects of grade and of aging and cooking on the distribution of nitrogen constituents have been made on other samples, and results of these studies will be reported later.

The content and distribution of arginine, leucine, tyrosine and histidine in prime and good grade beef rib steaks, NPN fractions and drippings are presented in Tables 2 and 3. The values are expressed in mg. of amino acid in the total cut, NPN fractions and drippings, and in the per cent of the total amino acid present in the cut. When calculated as per cent of the amino acid in the crude protein, the values check well with those found in the literature.

Tables 2 and 3 show that less than 1% of the total arginine, leucine and tyrosine is present in the drippings and less than 3% is present in the NPN fraction.

Aging of the samples had little effect on the arginine, leucine and tyrosine content of the samples. More arginine was found in the drippings after aging the good grade sample for 2 weeks. The NPN fraction contained more arginine, leucine and tyrosine after the samples were aged for 2 weeks. No striking differences in the relative increases were noted for these 3 amino acids, however.

Leucine and tyrosine apparently are present in bound form in the NPN fraction and drippings, since hydrolysis of these fractions resulted in increased amounts of microbiologically available leucine and tyrosine.

TABLE 2

The content and distribution of certain amino acids in prime rib steaks before and after aging [1]

Sample description	Arginine		Leucine		Tyrosine		Histidine			
							S. faecalis		*L. mesenteroides*	
	Total mg.	%	Total mg.	%	Total mg.	%	Total mg.	%	Total mg.	%
	Unaged samples									
Meat (raw)	7300	100	7610	100	3200	100	3770	100	3730	100
Meat (cooked)	6200	85	7740	102	3300	103	3180	84	3520	94
Drippings (unhydrolyzed)	11.5	0.16	4.1	0.05	2.5	0.08	34.1	0.91	7.9	0.21
Drippings (hydrolyzed)	15.3	0.21	12.9	0.17	4.8	0.15	266.7	7.1	231.3	6.2
NPN, raw (unhydrolyzed)	92.5	1.3	13.2	0.17	13.7	0.43	92.1	2.4	53.1	1.4
NPN, raw (hydrolyzed)	64.5	0.90	71.5	0.94	73.0	2.3	966.5	26	1011	27
NPN, cooked (unhydrolyzed)	63.3	0.87	15.2	0.20	10.1	0.32	51.3	1.4	37.7	1.0
NPN, cooked (hydrolyzed)	44.1	0.60	77.9	1.0	58.4	1.8	822.5	22	864.5	23.2
	Aged samples									
Meat (raw)	7000	100	8640	100	3600	100	4280	100	4120	100
Meat (cooked)	8320	119	8300	96	3600	100	3360	79	3940	96
Drippings (unhydrolyzed)	12.7	0.18	7.6	0.09	3.9	0.11	58.4	1.4	11.3	0.27
Drippings (hydrolyzed)	22.2	0.32	19.4	0.23	7.5	0.21	200.7	4.7	189	4.6
NPN, raw (unhydrolyzed)	147.8	2.1	26.6	0.31	34.4	0.96	88.8	2.1	73.7	1.8
NPN, raw (hydrolyzed)	139.9	2.0	189.7	2.2	46.4	1.3	1070	25	1119	29
NPN, cooked (unhydrolyzed)	110.6	1.6	45.4	0.53	29.2	0.81	51.3	1.2	55.1	1.3
NPN, cooked (hydrolyzed)	167.4	2.4	152.7	1.8	55.5	1.5	945.7	22	1016	25

[1] The values are expressed as the total mg. in the edible portion of the steak and as the per cent of the total amino acid present in the raw steak.

TABLE 3

The content and distribution of certain amino acids in good rib steaks before and after aging [1]

Sample description	Arginine		Leucine		Tyrosine		Histidine			
							S. faecalis		*L. mesenteroides*	
	Total mg.	%	Total mg.	%	Total mg.	%	Total mg.	%	Total mg.	%
	Unaged samples									
Meat (raw)	4860	100	5080	100	2400	100	2250	100	2480	100
Meat (cooked)	4810	99	5070	100	2100	88	2320	103	2170	88
Drippings (unhydrolyzed)	16.4	0.34	3.7	0.07	2.0	0.08	28.1	1.5	7.5	0.30
Drippings (hydrolyzed)	10.5	0.22	9.4	0.19	4.6	0.19	172.8	7.7	179.5	7.2
NPN, raw (unhydrolyzed)	63.4	1.3	7.4	0.15	6.8	0.28	67.4	3.0	15.5	0.63
NPN, raw (hydrolyzed)	25.1	0.52	39.3	0.77	16.3	0.68	729.3	32.0	698.4	28
NPN, cooked (unhydrolyzed)	44.8	0.92	10.1	0.20	5.7	0.24	62.4	2.8	14.0	0.56
NPN, cooked (hydrolyzed)	40.4	0.83	55.5	1.1	23.4	0.98	543.4	24.0	540	22
	Aged samples									
Meat (raw)	4460	100	4980	100	2230	100	2450	100	2470	100
Meat (cooked)	4230	94.8	4550	91.4	2200	98.7	2350	96	2030	82
Drippings (unhydrolyzed)	21.4	0.48	5.9	0.12	2.6	0.12	36.2	1.5	7.5	0.30
Drippings (hydrolyzed)	35.8	0.80	10.3	0.21	4.2	0.19	165.3	6.8	164.2	6.6
NPN, raw (unhydrolyzed)	76.7	1.7	17.9	0.36	20.3	0.91	42.9	1.8	23.4	0.95
NPN, raw (hydrolyzed)	39.5	0.89	42.9	0.86	31.5	1.4	350.4	14	435.2	18
NPN, cooked (unhydrolyzed)	60.3	1.4	11.8	0.24	19.4	0.87	31.8	1.3	25.0	1.0
NPN, cooked (hydrolyzed)	42.0	0.94	29.7	0.60	23.7	1.1	529	22	526	21

[1] See footnote, Table 2.

The lysine content and distribution in the NPN extracts or drippings appeared to be unaffected by aging. Bound forms of lysine and glutamic acid were found in both the drippings and NPN extracts.

As much as 7.9% of the total histidine in the meat was found in the drippings after hydrolysis, and up to 32% was found in the hydrolyzed NPN fraction after hydrolysis (this accounts for approximately 26% of the total nitrogen in these extracts). Since these figures represent increases of 3- to 6-fold in the drippings and 15- to 29-fold in the NPN fraction above the values for the unhydrolyzed samples, the occurrence of carnosine or other histidine peptides was suggested. It is of interest to point out that Lyman and co-workers *(3)* showed that up to 25% of the total histidine in beef loin was extracted by 60% acetone, and the major portion was not available to *S. faecalis* or *L. mesenteroides* P-60 before acid hydrolysis of these extracts. The present experiments showed that carnosine was approximately 15-20% active for *S. faecalis* before hydrolysis, but carnosine showed little or no activity (approx. 2%) for *L. mesenteroides*. Theoretical quantities of histidine were found with both test organisms after hydrolysis of carnosine.*

That the two organisms used differ in their ability to utilize bound forms of histidine is apparent from the data presented in Tables 2 and 3. Here the values for histidine in the unhydrolyzed drippings and NPN fractions as determined with *L. mesenteroides* are lower than those determined with *S. faecalis*, but the values for the hydrolyzed fractions are the same with both organisms. Presence of carnosine could only account for 6- to 7-fold increases after hydrolysis with *S. faecalis* as the test organism. Since increases of over 15-fold were observed it is concluded that substances that contain histidine, other than carnosine, were present in the NPN fraction of the beef samples. Little or no increase was observed in the histidine content of the unhydrolyzed or hydrolyzed drippings or NPN fraction after aging of the meat for two weeks.

The presence of carnosine in beef has been well established by chemical isolation procedures and has been confirmed recently in our laboratories *(6)* using paper chromatographic techniques. The present results are in agreement with the chromatographic work in that the soluble histidine has been shown to be largely in the combined form.

Williams and Krehl *(8)*, working on the formation of carnosine by rat liver slices, measured the synthesis indirectly by assaying for histidine by the use of microbiological method with *L. mesenteroides* as test organism. They state that it appears unlikely that any other histidine-containing substance but carnosine was being estimated. It is evident that in our experiments any increase above 7-fold in the histidine activity after hydrolysis as measured with *S. faecalis* must be due to substances like, but other than, carnosine in the NPN fraction of the beef samples.

The increased amounts of arginine, leucine and tyrosine in the drippings and NPN fractions after aging the samples for two weeks indicates proteolytic enzyme activity. This is in agreement with the increase in free amino nitrogen during aging and as the result of cooking. It would be

*We are indebted to Dr. Emil Smith for providing the carnosine nitrate used in these studies.

of interest to further investigate this proteolytic activity under various conditions and to determine the relative rates of release of different amino acids and peptides by microbiological analysis. It would also be of interest to determine the nature of the short chain peptides in the drippings and NPN fractions which result in increased quantities of the amino acids as determined microbiologically after hydrolysis of the drippings and NPN fractions.

SUMMARY

The content and distribution of nitrogen and of arginine, leucine, tyrosine, histidine, glutamic acid and lysine in raw and cooked beef muscle, the drippings from the cooked meat and the non-protein nitrogen (NPN) fraction were determined before and after aging the samples for two weeks. Amino acid analyses were made on the drippings and NPN fractions before and after acid hydrolysis.

Aging in the cut increased the free amino acid nitrogen of raw rib steaks. Cooking caused a very marked decrease in the amount of soluble protein nitrogen present and resulted in the liberation of some free amino nitrogen. Only a very small proportion of the total nitrogen present in the raw meat was found in the drippings after broiling. This nitrogen was essentially all non-protein nitrogen, and a fairly high proportion was present as free amino nitrogen.

A greater percentage of arginine, leucine and tyrosine was found in the drippings and NPN fractions after two weeks' aging than in the paired steaks that were not aged. Less than 3% of the total amount of these amino acids in the meat was found in the drippings or NPN fraction.

Bound forms of leucine, tyrosine, glutamic acid and lysine are present in the drippings and NPN fraction of beef.

Up to 31% of the total histidine was found in the NPN fraction of the samples, and the nitrogen from histidine accounted for approximately 26% of the NPN in the extracts. On the basis of differential microbiological analysis with carnosine as a reference standard, it was concluded that, in addition to carnosine, other bound forms of histidine are present in the drippings and NPN fractions of beef muscle.

LITERATURE CITED

1. Hiner, R. L., Gaddis, A. M., and Hankins, O. G. *Food Technol.*, **5**, 223 (1951).
2. Husaini, S. A., Deatherage, F. E., Kunkle, L. E., and Draudt, H. N. Food *Technol.*, **4**, 313 (1950).
3. Lyman, C. M., Kuiken, K. A., and Hale, F. *J. Biol. Chem.*, **171**, 233 (1947).
4. Schweigert, B. S., Tatman, I. E., and Elvehjem, C. A. *Arch. Biochem.*, **6**, 177 (1945).
5. Schweigert, B. S., Guthneck, B. T., Kraybill, H. R., and Greenwood, D. A. *J. Biol. Chem.*, **180**, 1077 (1949).
6. Sidwell, C. G., and Doty, D. M. (Unpublished data, 1951).
7. Thompson, H. T., Schurr, P. E., Henderson, L. M., and Elvehjem, C. A. *J. Biol. Chem.*, **182**, 47 (1950).
8. Williams, H. M., and Krehl, W. A. *J. Biol. Chem.*, **196**, 443 (1952).

DETECTION OF WASHED EGGS BY CONDUCTIVITY MEASUREMENTS. I. TEST METHODOLOGY

RAYMOND HIXON,[a] R. J. CONNOLLY,[a] AND GEORGE F. STEWART
University of California, Davis, California

(Manuscript received December 17, 1953)

A large amount of work has been reported in the literature on the effects of washing on the keeping quality of shell eggs. Despite the successes reported by a few workers, it is generally agreed that cleaning eggs with aqueous solutions is extremely hazardous with respect to their storage life thereafter. In view of this fact, those storing eggs are beset with the difficulty of determining whether or not a given lot contains washed eggs. The method of Sharp *(1)* wherein the presence or absence of potassium or chloride ion is determined in a water extract of the shell is slow and tedious. Hence egg handlers have turned to the use of ultra-violet light. To the authors' knowledge, no data exist to evaluate this method. However, studies are in progress and we will report on its efficacy in a later paper.

In our search for a simple and accurate method for detecting washing, our attention was directed to a conductivity test being used in Australia. We are indebted to Dr. J. R. Vickery[b] for information on this method, especially with respect to the circuits and electrodes used.

EXPERIMENTAL

Eggs. Clean and dirty eggs were obtained from the University flock. In addition, dirty eggs were obtained from a commercial handler. The latter came from several farms and were of variable age when obtained.

Washing technique. For the trials reported here the eggs were hand washed in running tap water at 120°F. They then dried on cup flats in a well ventilated room. Except where indicated, they were then held at 60°F. and 80% relative humidity before being subjected to the conductivity test.

Conductivity apparatus. The setup was adopted from a design supplied by Vickery *(loc. cit.)*. This consisted of a constant voltage power supply, a rectifier, a voltage divider, a volt meter, an ammeter and an electrode probe. See Figure 1. In this unit the 115-volt ac line power is rectified by a half wave selenium cell protected by an eight-ohm resistor. The voltage regulation tube (VR 105) maintains a constant voltage (105 volts) by passing increasing amounts of current as the rectifier voltage increases above 105 volts (by virtue of the RI drop across the 2,000-ohm resistor). The 10,000-ohm potentiometer permits voltage regulation on the electrode probe. The voltmeter is positioned in parallel and the ammeter in series relative to the probe electrodes.

The probe consisted of 2 bright platinum wires (1.46 diameter) sealed 3.32 mm. apart and extending 1.5 mm. beyond the seal.

Test conditions. The electrodes are placed firmly against the egg and a drop of distilled water placed between them, or, alternately, they are plunged into a drop of distilled water placed on the egg and then held firmly against the the shell until

---

[a]Employed jointly by the Biological Sciences Division, Agricultural Marketing Service, USDA, and University of California. This work was financed under authority of the Agricultural Act of 1946 (RMA, Title II).

[b]Commonwealth Scientific and Industrial Research Organization, Homebush, New South Wales, Australia.

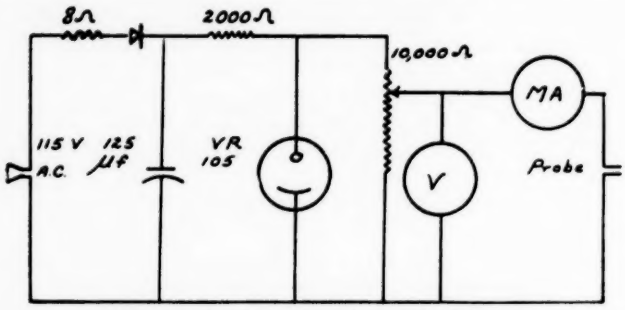


Figure 1. Electrical circuit for conductivity test.

the conductivity is read on the ammeter. On removal the electrodes are washed with distilled water; excess water is removed with absorbant paper.

RESULTS AND DISCUSSION

Several operating variables were tested in order to arrive at optimal test conditions. Specifically the effect of voltage input, extraction time, current application time, number of readings per egg, and number of eggs per test were studied.

Figure 2 shows the effect of probe voltage on the conductivity of extracts from washed and unwashed eggs (one test per egg). Although there were greater differences at the higher voltages, electrolysis and gas evolution caused wide fluctuations in the readings. Between 10 and 30 volts these effects were minimal and current readings stabilized within a short time. Most subsequent tests were therefore made at 15 volts.

Extraction time is of some importance, as is indicated by the data presented in Figure 3. In this trial the probe was placed in the drop after a designated time and allowed to remain until a relatively constant reading was obtained. More important is the time elapsed after the probe is placed in the extraction drop (Figure 4). It was found convenient and reasonably accurate to make the readings at 30 seconds. Most of those to be reported hereafter are for that time.

The effect of temperature on conductivity values is shown by the data in Table 1. From these data it would seem that temperature should be

TABLE 1

Effect of temperature on conductance of egg shell extracts[1]

Temperature, °F.	Conductance–ma.				Ratio
	Washed		Unwashed		
	No. eggs		No. eggs		
60	50	0.187	50	0.360	.52
70	50	0.176	50	0.338	.52
95	50	0.378	50	0.680	.54

[1]Commercial dirty eggs used; 60°F. lot not drawn from same sample of eggs as 70°F. and 95°F. lots. However, so far as known it was made up of eggs of same history. Test conditions: 15 volts; no prior extraction time; probe in extract 30 seconds before reading; two readings per egg.

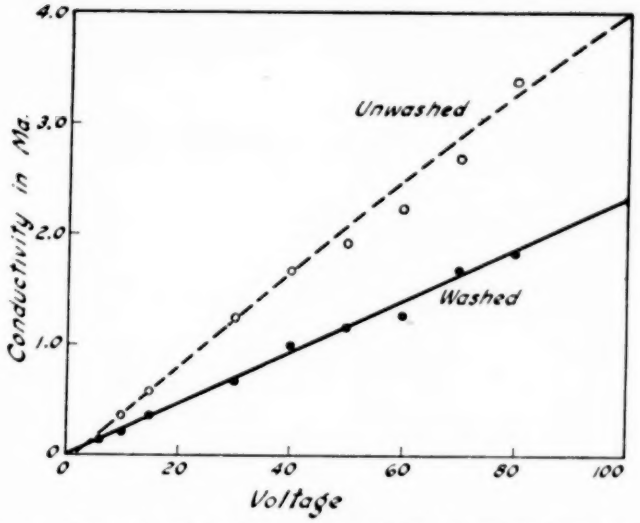


Figure 2. Effect of probe voltage on conductivity of extracts from washed and unwashed eggs. Twenty washed and 20 unwashed eggs were used; one reading per egg.

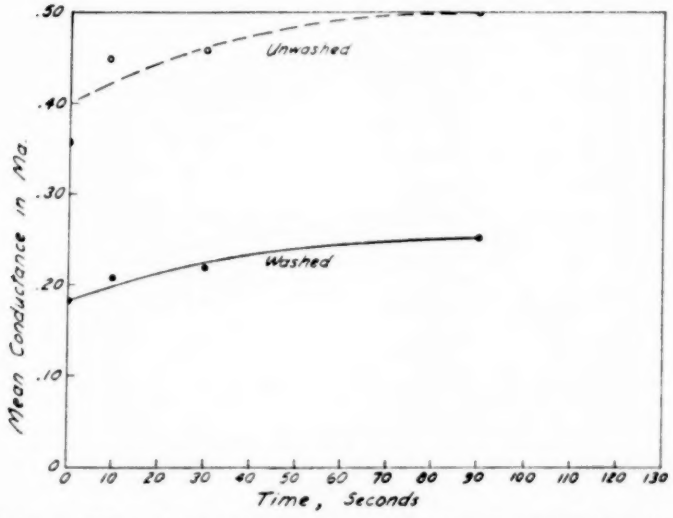


Figure 3. Effect of extraction time on conductivity of extracts of washed and unwashed eggs. Test conditions: 10 eggs per mean; 15 volts; reading taken 30 seconds after probe inserted.

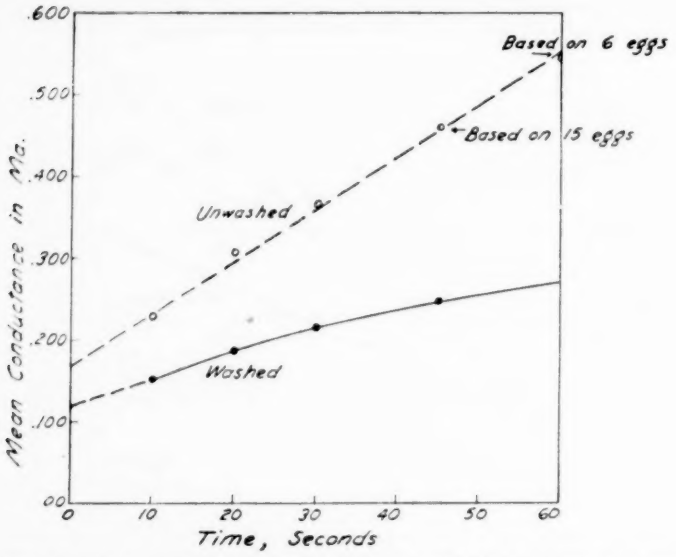


Figure 4. Effect of electrode contact time on conductance of egg shell extracts. Test conditions: 60 eggs per mean; 15 volts.

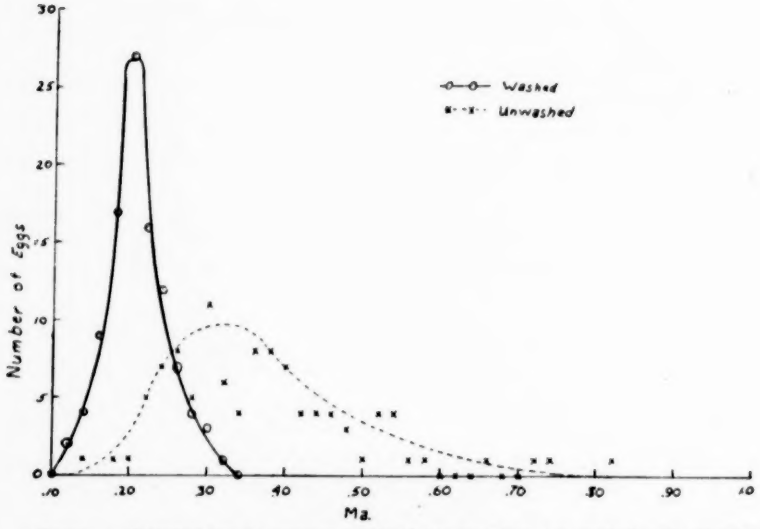


Figure 5. Distribution of average conductivity readings for washed and unwashed eggs. Test conditions: 15 volts; 30 seconds contact time; 5 readings per egg; 100 washed, 100 unwashed eggs.

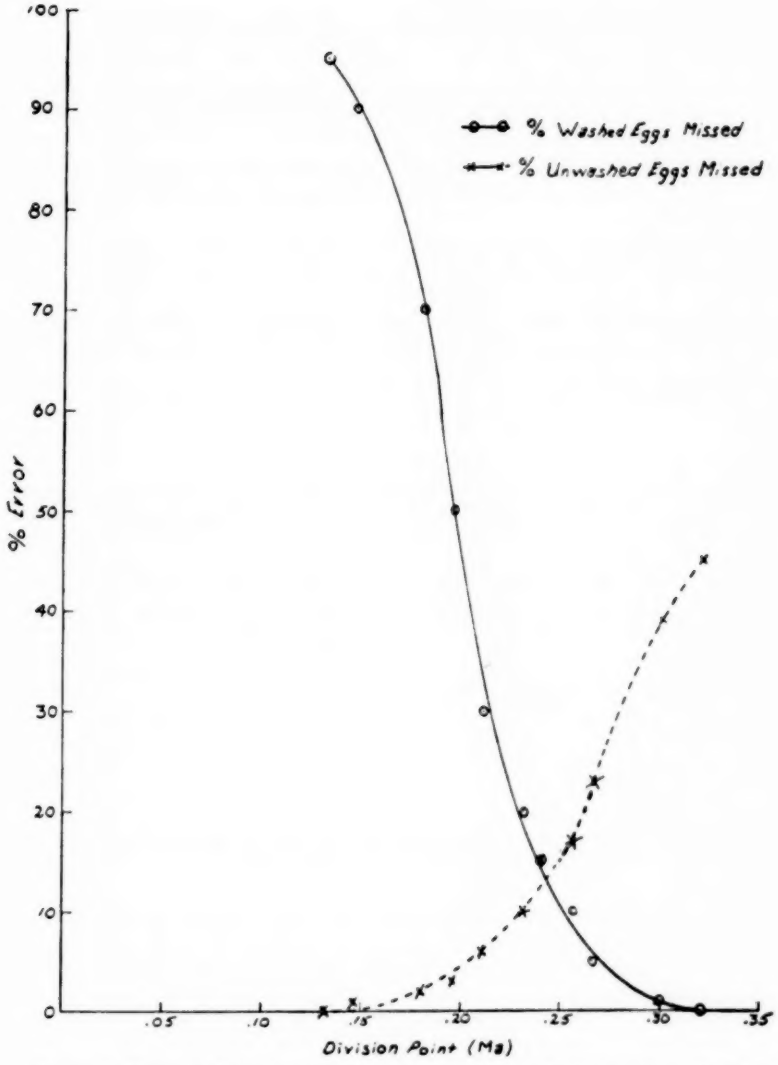


Figure 6. Errors in detecting egg washing at various conductance values. Based on 100 washed and 100 unwashed eggs; five readings per egg.

kept reasonably uniform for both eggs and instrument. There appears to be no preference among the 3 temperatures studied. We chose to make most subsequent tests at ± 60°F.

Intra- and inter-egg variations in conductivity were studied in a trial involving 100 washed and 100 unwashed eggs. Five readings at points around the small circumference per egg were made. The data obtained are shown in Figures 5, 6, and 7. From Figure 5 it appears that unwashed eggs have a much wider range of values compared to washed. The distribution curve is strongly skewed toward higher values. Washing tends to eliminate these high values; for washed eggs they show a fairly normal distribution.

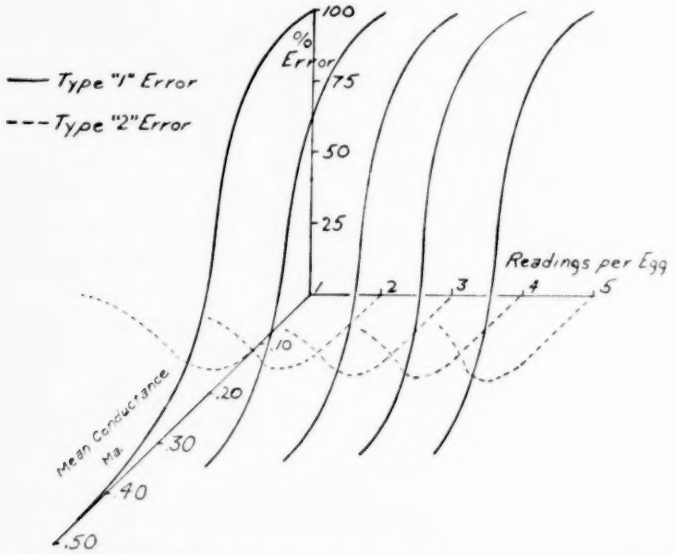


Figure 7. Effect of number of conductance readings per egg on errors in detecting egg washing. Based on 100 washed and 100 unwashed eggs.

The overlapping of the two distribution curves (from 0.12 to 0.35 ma.) means that readings falling in this range cannot be used accurately to identify washed eggs. In order to determine how accurately one might classify any egg as washed or unwashed, Figure 6 was constructed. Type I error occurs when one calls a washed egg unwashed; type II error calling unwashed eggs washed.

The errors shown are based on using various conductivities as arbitrary dividing points between washed and unwashed eggs. Thus, if 0.32 ma. is used as the dividing point, then none of the washed eggs would have been missed (% type I error); on the other hand, 45% of the unwashed eggs would have been missed (45% type II error). Minimal error for both washed and unwashed eggs occurs at approximately 0.24 ma.

The influence of numbers of readings per egg on the accuracy of the test is shown in Figure 7. This shows that increasing the number of readings from one to two per egg significantly decreases both type I and II errors. Additional readings have much less influence but are in the same direction.

Studies are under way to determine the applicability and limitations of this test under commercial conditions. They will be reported at a later date.

SUMMARY

Studies were made of a conductivity test aimed at detecting washed eggs. Important variables found to influence results included: (a) voltage input, extraction time, voltage application time, and egg temperature. Intra- and inter-egg variations were high, especially for unwashed eggs.

Large average differences between washed and unwashed eggs were found. However, a considerable overlap was found in the distribution curves for washed and unwashed eggs. Detection errors were determined for different cut-off points. The point of minimal error in detecting washed or unwashed was established.

LITERATURE CITED

1. SHARP, P. F. The detection of washed, abrased and oiled eggs. *Ind. Eng. Chem.*, **24**, 941 (1932).

STUDIES ON THE USE OF COATING FOR EXTENSION OF STORAGE LIFE OF FRESH FAJLI MANGO

A. N. BOSE AND G. BASU
College of Engineering and Technology, Bengal, India

(Manuscript received January 2, 1954)

In the Malda district of West Bengal, mangoes of the Fajli variety form the principal cash crop. The value of mango produced annually runs into over 2 crores of rupees. But Fajli mangoes, after they have been picked from the trees at the proper stage of maturity, can be kept only for a week or two. Limited storage life has resulted in the waste of huge amounts of this valuable fruit. Methods for extending the storage life even by a few weeks are urgently needed not only to save a valuable food material from wastage, but also to stabilize the economic life of the growers.

The reasons for the decay of fresh fruits during storage are both pathological and physiological. The pathological decay is caused by the bacteria and fungi with which the fruit is contaminated. Physiological decay is caused by catabolic activities, chiefly indicated by respiration—in which the sugar, acid, protein, oil etc. in the fruit are destroyed to maintain life of the fresh fruits—and by transpiration—i.e. loss of water which accompanies respiration. The effect of respiration and transpiration is loss of food-value, flavor, appearance and weight, the net result of which is to render the fruit unmarketable. However normal respiration cannot be completely cut off because anaerobic respiration then takes place, producing chemical by-products which cause off-flavors and death of the fruit.

Various workers like Cheema and Gandhi *(3)*, Banerjee, Karmarkar and Row *(1)* and Cheema and Dani *(2)* have studied the preservation of different varieties of mangoes in cold-storage at different temperatures. In a very exhaustive work Cheema, Karmarkar and Joshi *(4)* studied cold-storage behavior of different varieties of mangoes available in India, but they did not study the Fajli variety of mango from the Malda District of West Bengal. Moreover, all the studies mentioned above were conducted with the untreated mangoes.

Control of the respiration and transpiration process by means of a suitable coating, has been widely practiced in the U.S.A. for the preservation of oranges with great success.

The object of the investigation reported here was to find a method of a preservation, by proper coating of the fruit, which would restrict respiration, and thus increase the storage life of fresh mangoes with minimum decay or physiological deterioration.

EXPERIMENTAL

Mangoes used in these experiments were of Fajli variety and obtained from Malda district in West Bengal. As the preliminary experiments showed that unless the mango of Fajli variety is picked at the stage corresponding to the maturity stage B, according to Cheema and Dani *(2)*, it does not ripen properly, the experiments under this investigation were conducted with mangoes of stage B maturity. Paraffin of low

melting point (m.p. 75-80°C. or 167-176°F.) was used for coating the mangoes under investigation.

To obtain different thickness of the paraffin coating the following procedure was adopted: the mango to be coated was dipped for 10 seconds either in (a) molten paraffin at 80°C.(176°F.) or (b) 50% solution of paraffin in petroleum ether at 25°C. (77°F.).

The dipping period of 10 seconds was chosen because it was observed that with molten paraffin it was the minimum time necessary to assure uniform coating without causing any appreciable heat damage to the fruit. With solutions of paraffin, the duration of contact should be as short as possible to prevent the action of petroleum ether on the fruit tissue.

RESULTS AND DISCUSSION

The effect of the coating on respiration was measured. Results on the measurement of respiration intensity are given on Table 1.

It will be observed from Table 1 that proper coating with paraffin will reduce the rate of respiration without completely stopping it. Respiration intensities at the lower temperatures at which mangoes may be stored have not been determined, but it is believed that values of respiration intensities at 37°C.(98.6°F.) fairly indicate the trend.

TABLE 1

Respiration intensity of Fajli mango at 37°C.(98.6°F.)

Stage of maturity (according to Cheema and Dani)	Treatment	Respiration intensity (mgm. of CO_2/hr./kg.)
1. B	Untreated	9.7
2. B	Dipped in molten paraffin at 80°C. for 10 seconds	2.2
3. B	Dipped in a 50% soln. of paraffin in petroleum ether at 25°C. for 10 seconds	8.04

TABLE 2

Loss in weight of Fajli mango stored at 25°C.(77°F.) and 90% relative humidity

Treatment	Hours in storage	Loss in weight (as percentage of the initial weight of the fruit)
1. Untreated	0	.....
Untreated	48	3.06
Untreated	96	6.35
Untreated	144	9.42
Untreated	192	11.00
2. Dipped in paraffin bath (80°C.) for 10 seconds	0	.....
Dipped in paraffin bath (80°C.) for 10 seconds	48	0.36
Dipped in paraffin bath (80°C.) for 10 seconds	96	0.98
Dipped in paraffin bath (80°C.) for 10 seconds	144	1.80
Dipped in paraffin bath (80°C.) for 10 seconds	192	2.33
3. Dipped in 50% soln. of paraffin in petroleum ether at 25°C.	0	.....
Dipped in 50% soln. of paraffin in petroleum ether at 25°C.	48	1.56
Dipped in 50% soln. of paraffin in petroleum ether at 25°C.	96	2.80
Dipped in 50% soln. of paraffin in petroleum ether at 25°C.	144	4.35
Dipped in 50% soln. of paraffin in petroleum ether at 25°C.	192	5.20

The effect of coating on the rate of transpiration, as indicated by the loss in weight of the fruit, was also determined and results are given in Table 2.

Thus it will be observed from Tables 1 and 2 that proper coating with paraffin will materially reduce the rates of respiration and transpiration of Fajli mango and should, therefore, appreciably increase the storage life of the fruit. But whether such lowering of aerobic respiration will stimulate anaerobic respiration and lower the organoleptic qualities and appearance of the fruit can only be known by storage

TABLE 3

Storage at 2.2°C.(36°F.) and 90% relative humidity

Treatment	Days in storage	Observations
1. Untreated	0	Maturity stage B
Untreated	6	Skin has dried appearance. Otherwise unchanged
Untreated	12	Skin wrinkled. Otherwise unchanged
Untreated	15	Skin very wrinkled. Ripe smell but not normally ripe otherwise. Juice oozing from the stern end. Black patches on stern end
Untreated	18	Rotting advanced without proper ripening
2. Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	0	Maturity stage B
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	6	Unchanged
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	12	Unchanged
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	18	Few black spots on the skin. Otherwise unchanged
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	21	Black spots extended in size and number
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	24	Juice oozing from the stem. Ripe smell. Not normal ripening
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	28	Rotting advanced
3. Dipped in a 50% soln. of paraffin for 10 seconds	0	Maturity stage B
Dipped in a 50% soln. of paraffin for 10 seconds	6	Unchanged
Dipped in a 50% soln. of paraffin for 10 seconds	12	Unchanged
Dipped in a 50% soln. of paraffin for 10 seconds	18	Unchanged except few black spots on skin
Dipped in a 50% soln. of paraffin for 10 seconds	21	Softness. Black spots extended
Dipped in a 50% soln. of paraffin for 10 seconds	24	Juice oozing out from the stem end. No normal ripening
Dipped in a 50% soln. of paraffin for 10 seconds	28	Advanced spoilage

experiments. It was observed that at room temperatures coating had no beneficial effect on storage-life, in fact, it caused some unnatural changes. Hence, storage at lower temperatures i.e., at (a) 2.2°C.(36°F.) (R.H. 90%) and (b) 12.8°C.(55°F.) (R.H. 90%) were tried. Results are shown in Tables 3 and 4.

Table 3 shows that in storage at a temperature 2.2°C.(36°F.) the paraffin coating has very little value in extending the storage-life of mangoes, and the defects may be mainly due to too low a temperature, which generally adversely affects tropical fruit like Fajli mango. In another series of experiments a relatively higher temperature, i.e., 12.8°C.(55°F.), was used for storage temperature. Results are given in Table 4.

It will be observed from Table 4 that Fajli mangoes of maturity B (according to Cheema and Dani) when coated by dipping in a paraffin bath at 80°C.(176°F.) for 10 seconds, can be stored at 12.8°C.(55°F.) (R.H. 90%) for 42 days without any deterioration. Mangoes so treated ripen normally.

TABLE 4

Storage at 12.8°C.(55°F.) and 90% relative humidity

Treatment	Days in storage	Observations
1. Untreated	0	Maturity stage B
Untreated	6	Shrivelled appearance
Untreated	12	Juice oozing out from stem end and blackening
Untreated	15	Advanced spoilage. Discarded
2. Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	0	Maturity stage B
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	6	No change
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	12	No change
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	18	No change
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	24	No change
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	30	No change
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	32	No change
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	38	Ripe smell. Softens a little
Dipped in paraffin bath at 80°C. (176°F.) for 10 seconds	42	Ripe smell, softens. Appears properly ripe
3. Dipped in a 50% soln. of paraffin for 10 seconds	0	Maturity stage B
Dipped in a 50% soln. of paraffin for 10 seconds	6	No change
Dipped in a 50% soln. of paraffin for 10 seconds	12	No change
Dipped in a 50% soln. of paraffin for 10 seconds	18	Appearance alters. (Shrunken) spoilage starts
Dipped in a 50% soln. of paraffin for 10 seconds	24	Spoilage advanced
Dipped in a 50% soln. of paraffin for 10 seconds	26	Discarded

SUMMARY

Respiration intensity at 37°C.(98.6°F.) for Fajli mangoes coated by (a) dipping in paraffin bath at 80°C.(176°F.) for 10 seconds and (b) dipping in 50% solution of paraffin in petroleum ether for 10 seconds, was found to be 2.2 and 8.04 mgm. of CO_2/hr./kg. as compared to 9.70 for the untreated. The mangoes were at the stage of maturity at which they are normally picked from the tree (maturity stage B of Cheema and Dani).

Coating with paraffin by the methods (a) and (b) above also reduces transpiration as measured by loss in weight of fruit in storage. Loss in weight was 2.34% of the initial weight for the mango coated with paraffin by method (a) compared to 11.00% for the untreated mango when stored at 25°C.(77°F.) and 90% R.H. for 192 hours.

The Fajli mango, coated with paraffin by method (a), was found to stay in good condition for 42 days when stored at 12.8°C.(55°F.) and 90% R.H. while the untreated mango rotted in 14 days. All mangoes were initially of the maturity stage B (according to Cheema and Dani).

Acknowledgment

The authors are grateful to the Mango Improvement Committee, Malda, West Bengal, for financing a scheme of which this investigation forms a part.

LITERATURE CITED

1. Banerjee, B. N., Karmarkar, D. V., and Row, G. R. *Agric. Live-stock Ind.* **4**, 36 (1934).
2. Cheema, G. S., and Dani, P. G. Dept. Agric., Bombay. *Bull No. 170* (1934).
3. Cheema, G. S., and Gandhi, S. R. *Agric. J. Ind.*, **21**, 403 (1926).
4. Cheema, G. S., Karmarkar, D. V., and Joshi, B. M. Imp. Council of Agric. *Resh. Misc. Bull.* No. 21.

A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF BEEF DEHYDRATION. III. INFLUENCE OF PRE-COOKING*

EARL AUERBACH, H. WANG, VIRGINIA BATES, D. M. DOTY, AND H. R. KRAYBILL

American Meat Institute Foundation and Department of Biochemistry, The University of Chicago, Chicago, Illinois,

(Manuscript received January 6, 1954)

Research on commercially dehydrated meat produced prior to and during World War II had indicated that regardless of the method of dehydration, the best products were obtained by cooking, grinding, and drying below 70°C. as rapidly as possible *(1, 2, 3, 4)*. Recently certain differences were established between the dehydration and rehydration characteristics of raw and steam pre-cooked, unground beef tissue *(5)*, but the data indicated little desirability for dehydrating meat in its cooked state. Since then, the use of electrolytic pre-treatment has been shown to favor dehydration and a subsequent degree of rehydration *(6)*.

The series of experiments reported in this paper were undertaken for the purpose of determining the best temperatures of pre-cooking and dehydration, with or without electrolytic treatment, that would yield a product of high rehydratability.

MATERIAL AND METHODS

Cylinders (½-inch in diameter by 2 inches in length and weighing 2-3 g.) of *Biceps femoris* from a commercial grade carcass were pre-cooked to internal temperatures of 60°, 76.6°, and 93.3°C. in a beef tallow bath and dried for 16 hours at 45°C. under vacuum and at 70°C. in an air oven. Duplicate cylinders were electrolyzed after pre-cooking and prior to dehydration. Another set of experiments consisted of cooking large blocks of tissue (300-400 g.) and then removing the cylinders for further treatment.

The method for pre-cooking in a beef tallow bath was used because it is rapid, affords good temperature control, and results in small loss of soluble constituents of the tissue. Thermocouples were used for temperature measurements. Both cylinders and blocks were allowed to remain in the heated tallow bath until the desired internal temperature had been reached. The temperature of the tallow bath was approximately 10 degrees above the desired final internal temperature of pre-cooking.

Methods for rehydration, moisture determination, electrolysis, histological, and histochemical treatment were the same as previously used *(5)*.

EXPERIMENTAL RESULTS AND DISCUSSION

Histological and histochemical observations. Muscle fiber shrinkage of raw samples was slight as a result of pre-cooking in an oil bath. This may have been due to the fact that water loss was mainly from endomysial and perimysial spaces, rather than from muscle fibers. Histological observations substantiated this. There was a decrease in endomysial space

*Journal Paper No. 86, American Meat Institute Foundation. This paper reports research sponsored by the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned No. 461 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as reflecting the views or endorsement of the Department of Defense.

in dehydrated and rehydrated pre-cooked samples which was considered higher than in similarly treated raw samples. At the same time, the pre-cooked samples, both dehydrated and rehydrated, showed a lesser degree of muscle fiber merging than did raw samples.

The possibility existed that oil penetration into the samples during cooking may have had some influence in dehydration. However, frozen sections of oil-cooked samples stained with either Nile blue sulfate or Sudan IV *(7)* did not reveal fat penetration.

Duplicate sections that were examined histologically were subjected to the Carere-Comes orange technique for potassium. Recent papers *(5, 6)* have shown that potassium is present in the muscle fibers, concentrated particularly at the cross-striations. Dehydration of raw tissue will cause this mineral to translocate to the periphery of the cell by the outward movement of cell fluids. Pre-cooking of muscle tissue caused a similar but less pronounced translocation. Electrolysis removed the potassium from the muscle cells, as evidenced by both staining procedures and analysis of the electrolytic bath. Sections of samples dehydrated after pre-cooking and electrolysis showed little difference in potassium distribution from sections of pre-cooked electrolyzed samples before dehydration. Comparison of pre-cooked tissue and raw tissue, similarly treated, indicated a marked similarity in potassium distribution; thus, any differences in the dehydration and rehydration behavior of the pre-cooked samples, as compared to raw samples, cannot be related to the distribution of potassium or probably any other inorganic ions.

Moisture data. Preliminary experiments on pre-cooking of individual cylinders indicated a variability of residual moisture content after dehydration. This residual moisture was as high as 26.7% in the non-electrolyzed samples and 17.9% in the electrolyzed samples. As a result, it was decided to pre-cook a large block of muscle tissue to an interal temperature of 76.6°C. and then remove the cylinders (block cylinders) for further treatment. These block cylinders and individual cylinders pre-cooked at 76.6°C. were then dehydrated. The block cylinders dehydrated at 45°C. contained 6.2 ± 0.56* per cent moisture and those dehydrated at 70°C. contained 0.2 ± 0.23* per cent. Individually pre-cooked cylinders dehydrated at 45°C. contained 15.1 ± 2.0* per cent moisture while those dehydrated at 70°C. contained 7.8 ± 1.7* per cent. Thus, the cylinders cut from pre-cooked blocks dehydrated to a lower moisture content at both temperatures of dehydration than did the individually pre-cooked cylinders. One might conclude that this difference is related to the fact that the cylinders cut from pre-cooked blocks received more heat than did the individually pre-cooked cylinders.

More extensive experiments were done to determine the influence of cooking temperature and electrolysis on the moisture changes and histological properties of cylinders of beef dehydrated at different temperatures. The data (Table 1) confirm those given above in that for non-electrolyzed samples the moisture content of the dehydrated cylinders from cooked blocks of meat was considerably lower than that of cylinders individually pre-cooked. This was true for all temperatures of pre-cooking and for

*Mean for at least 3 samples and the standard error.

both dehydration temperatures. It also was true for electrolyzed samples pre-cooked to 60°C. but not for those pre-cooked to 76.6°C. and 93.3°C. For non-electrolyzed samples the muscle fiber diameter of beef in the block cylinders was reduced more by dehydration than it was in the individually pre-cooked cylinders. Again this was true for all temperatures of pre-cooking and for both temperatures of dehydration. In the case of electrolyzed samples it was true only for samples pre-cooked to 60°C.

As for raw samples *(6)*, electrolysis of the cooked meat resulted in a slight increase in moisture content. This was somewhat more pronounced for the block cylinders. However, the muscle fiber diameter was not influenced consistently by electrolysis. The electrolyzed samples rehydrated to a higher moisture content than did corresponding samples not electrolyzed. Also, the block cylinders usually absorbed more water during rehydration than did the individually pre-cooked cylinders. The greatest water absorption was exhibited by electrolyzed block cylinders pre-cooked to 76.6°C. and dehydrated at 70°C. The muscle fiber diameter usually was increased slightly by rehydration. This was more pronounced in the block cylinders than in the individually pre-cooked cylinders and was found more consistently in samples pre-cooked to 60°C. and 76.6°C. than in samples pre-cooked to 93.3°C. Temperature of dehydration and electrolysis had

TABLE 1

The moisture content and muscle fiber diameter in cylinders of pre-cooked beef after different treatments

Internal temperature of pre-cooking			60°C.		76.6°C.		93.3°C.	
Sample treatment	Method of pre-cooking	Dehydration temperature	Moisture %	Diameter μ	Moisture %	Diameter μ	Moisture %	Diameter μ
Pre-cooked	In cylinder	.....	70	37	67	35	62	37
	In block	.....	69	38	68	32	59	38
Pre-cooked, Electrolyzed	In cylinder	.....	74*	44	74	36	67	36
		.....	73*	39	69	32	63	37
	In block	.....	80*	37	72	28	68	31
		.....	78*	31	75	30	67	33
Pre-cooked, Dehydrated	In cylinder	45°C.	21.6	25	26.7	30	21.1	32
		70°C.	16.2	23	13.1	30	21.7	29
	In block	45°C.	5.8	23	2.0	22	3.8	28
		70°C.	11.6	24	4.2	23	3.7	29
Pre-cooked, Electrolyzed, Dehydrated	In cylinder	45°C.	17.9	27	—0.9	31	—2.9	28
		70°C.	17.0	29	9.6	25	6.8	34
	In block	45°C.	4.3	30	15.0	21	2.0	26
		70°C.	10.0	26	1.2	25	7.2	31
Pre-cooked, Dehydrated, Rehydrated	In cylinder	45°C.	27.8	29	38.2	29	35.0	31
		70°C.	28.9	28	23.5	31	35.1	30
	In block	45°C.	27.1	25	42.8	26	22.9	30
		70°C.	25.7	27	33.3	29	42.4	33
Pre-cooked, Electrolyzed, Dehydrated, Rehydrated	In cylinder	45°C.	46	30	57	33	46	31
		70°C.	68	31	44	29	51	31
	In block	45°C.	57	33	67	29	46	29
		70°C.	60	26	71	29	57	30

* Values for duplicate cylinders.

no consistent influence on the extent of muscle fiber swelling during rehydration.

Inspection of the data suggested that there might be a correlation between the moisture content and fiber diameter of the pre-cooked samples after the various treatments used. The calculated correlation coefficient between moisture content and muscle fiber diameter for the data in Table 1 is 0.612, which is quite close to that previously reported for raw meat *(6)*.

SUMMARY

Samples of *Biceps femoris* were pre-cooked in beef-tallow at three different internal temperatures, and dehydrated at two different temperatures. These samples were divided into two groups: electrolyzed and non-electrolyzed.

The data indicate that the best internal temperature of pre-cooking was 76.6°C. for only the electrolyzed, block cylinder group. This was based on the degree of subsequent rehydration. Electrolyzed block cylinder samples dehydrated at 70°C. rehydrated to a higher level of rehydration than those similarly treated samples dehydrated at 45°C., regardless of cooking temperature.

Statistical analysis of the samples revealed a calculated correlation coefficient of 0.612 for moisture content—fiber diameter values.

In dehydrating unground beef tissue, there is no marked advantage of pre-cooked over raw meat.

LITERATURE CITED

1. Bate-Smith, E. C. Dehydration. Section II (Meat and Meat Products). Part I. Drying of Meat. United Kingdom *Progress Report*, No. 17, pp. 491-9, January 1943.
2. Dunker, C. F., Hankins, O. G., and Bennett, O. L. Producing dehydrated meats by vacuum-rotary, plate-vacuum, and kettle-cabinet methods. *Food Research*, 10, 445-456 (1945).
3. Hankins, O. G., Dunker, C. F., and Bennett, O. L. Producing dehydrated meats by drum-bacinet, retort-cabinet, and other methods. *Ibid.*, 11, 1-13 (1946).
4. Kraybill, H. R. The dehydration of meat. *Proc. Inst. Food Technol.*, 90-94 (1943).
5. Wang, H., Andrews, F., Rasch, Ellen, Doty, D. M., and Kraybill, H. R. A histological and histochemical study of beef dehydration. I. Rate of dehydration and structural changes in raw and cooked meat. *Food Research*, 18, 351-359 (1953).
6. Wang, H., Auerbach, Earl, Bates, Virginia, Andrews, F., Doty, D. M., and Kraybill, H. R. A histological and histochemical study of beef dehydration. II. The influence of carcass grade, aging, muscle, and electrolysis pre-treatment. *Food Research* 19, 154 (1954).
7. Wang, H., Rasch, E., Bates, V., Beard, F. J., Pierce, J. C., and Hankins, O. G. Histological observations on fat loci and distribution in cooked beef. *Ibid.*, 19, 314-322 (1954).

SCLEREID DEVELOPMENT AND THE TEXTURE OF BARTLETT PEARS

CLARENCE STERLING
Department of Food Technology, University of California, Davis, California

(Manuscript received January 11, 1954)

Many varieties of pears, particularly the so-called "winter pears," are characterized by an abundant development of *sclereids* ("grit" or "stone" cells with thick, lignified walls) among the parenchymatous or thin-walled cells of the flesh. The presence of such sclereids, particularly in closely-knit "nests" or clusters, conveys a pronounced gritty texture to the flesh of the pear. Lignified, thick-walled sclereids are also responsible for the toughness of date skins and the hardness of almond pits and nut shells. An overall picture of the development of the sclereid clusters in pear in terms of chemical analysis has been presented by Crist and Batjer *(8)*. Their paper also reviews previous studies and presents extensive cultural and storage treatments which were carried out to determine causative aspects of sclereid formation.

In terms of chemical composition (percentage of lignin and cellulose on a dry weight basis) and microscopic observation, Crist and Batjer *(8)* report that the sclereids of pear decrease in number during fruit ripening. Such an observation would indicate that delignification and reduction of cell wall thickness occur in the sclereids of the maturing fruit. However, their microscopic account does not give a detailed picture of sclereid development nor does it seem to substantiate their conclusions regarding wall-thinning and delignification.

If it can be shown microscopically that there is a decrease in the number of sclereids via wall-thinning and loss of lignin during maturation of the pear fruit, not only would the finding be of great theoretical interest, but it would also be of considerable significance in food research. Consequently, the present study has been undertaken to ascertain microscopically changes in fruit texture and sclereid structure in a soft-fleshed pear. Degradative changes of the sclereids should be evident in such a pear if they occur at all during ripening.

MATERIALS AND METHODS

Bartlett pears were collected in 1951 from the time of flowering (April 9) to fruit fall (August 27) in Campbell, Santa Clara County, California. Some pears were stored at room temperature for one week (September 2) after fruit fall. Whole young fruits were taken first, and wedges from the mid-region (at the level of the seeds) were taken later as the fruits enlarged. These samples were killed and preserved in Randolph's fluid *(11)* and later embedded in paraffin after ethanol-xylol dehydration. Sections were cut at 10 μ to 15 μ and stained with tannic acid-ferric chloride and safranin for microscopic study. Phloroglucinol-HCl solution was used as a spot test for the presence of lignin *(11)*.

Morphological Development of the Pear Fruit

As in apple, quince, hawthorn, and related species of plants with pome fruits, the fruit of the pear is composed of a five-parted seed-bearing ovary in the center, sur-

rounded by and united with a thick fleshy region which is derived from a so-called "floral tube." The morphological construction of this floral tube has been variously interpreted by botanists, and a comprehensive review of their concepts has been given by Douglas *(9)*. It is this extra-ovarian floral tube which forms the bulk of the flesh of the pome fruit and in which sclereid formation takes place.

During the 20 weeks between flowering and fruit abscission, increase in diameter of the fruit follows a typical S-shaped growth curve. Average diameters at bi-weekly intervals are presented in Table 1. It may be seen that there is a slight slackening of the rate of growth during the period from 8 to 12 weeks after flowering. A similar depression of the growth rate was found by Mitchell *(14)*, and it also appears to be a consistent feature of the development of stone fruits *(12, 18, 26)*. Gross anatomical changes during development have been detailed by Mitchell *(14)*, with whose findings the writer's results substantially agree.

TABLE 1

Average diameter of Bartlett pear during development

Age in weeks	Diameter in mm.
0 (flower)	2
2	6
4	11
6	17
8	24
10	30
12	36
14	45
16	56
18	62
20	65

Tissue Development

At flowering (Figure 1), the floral tube region of the young fruit consists of a single layer of epidermal cells on the outside, inside this another layer, the hypodermal layer, which is also one cell thick, followed by a zone of about 25-30 layers of parenchymatous cells which will form the mass of the fruit flesh. At this stage, most of the parenchyma cells are small (15 μ to 20 μ in diameter) and thin-walled, relatively equal in size, and alike in shape (isodiametric). They divide actively in various random planes.

All figures are cross sections of fruit of Bartlett pear. Ovary is located toward base of page in each figure. All × 92.

Figure 1. Portion of ovary and floral tube regions at time of flowering. Ovary tissues are deeply staining at lower left.

Figure 2. Portion of interior of fruit at 2 weeks after flowering. Note radiating cell rows from ovary outward and initiation of concentric divisions above young sclereid cluster near bottom. Elsewhere the first sclereids are differentiating.

Figure 3. Portion of surface of fruit at 2 weeks after flowering. Substomatal air space developing (at arrow) in hypodermis, which is 2-4 layers thick here. Isolated sclereids are being initiated in outer flesh.

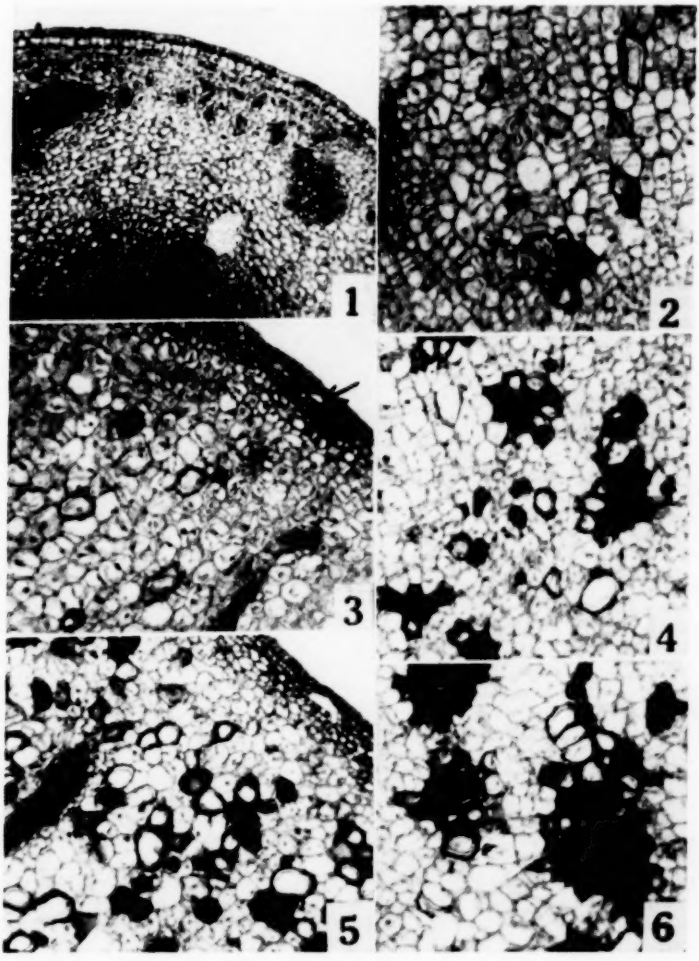
Figure 4. Portion of inner flesh at 4 weeks after flowering. Sclereid clusters are becoming larger, and new sclereids are being differentiated. Concentric divisions are more noticeable (clusters at lower left and middle top).

Figure 5. Portion of surface of fruit at 4 weeks after flowering. Substomatal chamber becoming wider. Note decrease in size of hypodermal cells. No sclereids have differentiated yet in sub-hypodermal region.

Figure 6. Portion of inner flesh of large fruit at 4 weeks. Concentric divisions and rows radiating from cluster are quite obvious.

No intercellular spaces are present, but various small branches of young vascular bundles may be found among these cells. Two of the 10 principal vascular bundles of the young fruit are shown in Figure 1.

"Skin." After fertilization, active cell division and cell enlargement take place. Although Mitchell *(14)* has stated that the epidermal layer remains single, experiencing only radial divisions, the specimens of the writer show numerous tangential divisions in this layer (Figures 7, 10, 13). The corky surface of many russet pears, as well as the corky lenticel areas of the Barlett pear, are formed as a result of active tangential cell divisions in a layer of cork cambium, which is often epidermally produced. Where the epidermis is maintained as a single layer, the writer can agree with Mitchell that the epidermal cells continue "to divide throughout the growing season with the continued enlargement of the fruit." Hence, at maturity, the epidermal cells are only slightly larger than at the time of flowering, despite the more than thirty-fold increase in fruit circumference (*cf.* Figures 1, 14).



Formation of stomata does not occur until after flowering (compare Figures 1 and 2). Continued tangential growth at the fruit surface gradually broadens the stomatal regions, which become wider and wider and eventually are pulled open (Figures 3, 5, 7, 13, 14). On the floor of the substomatal chamber, a cork cambium usually produces a suberized zone of cells *(13, 15, 30)* which can seal off the interior of the fruit from excessive evaporation (Figures 13, 14). Later, about 14-16 weeks after flowering, the outermost cells, which apparently remain alive, can extend outwardly as callus-like protuberances *(30)* (Figure 14). Where no corky tissue is formed, a thickened cuticle serves as a water barrier. Ordinarily, unlike the cuticle of apple *(3, 24, 25)* and some winter pears *(30)*, the cuticle of Bartlett pears is entirely superficial, being located on the surface of the outer tangential walls of the epidermal cells. However, where cells of the epidermis are pulled apart during growth, the cuticle may penetrate inwardly between the separated cells to the outermost hypodermal layer *(14, 15)*. In one case, the cuticle occurred as a peg-like projection to the second outermost hypodermal layer.

During fruit growth, the hypodermal layer divides by both tangential and radial divisions to produce a sub-epidermal zone of "collenchyma" cells, 4-6 layers thick, filled with some phlobaphene-like type of secretion (Figures 3, 5, 7). These cells resemble parenchyma cells but are smaller at maturity and have thickened walls, giving considerable strength to the so-called "skin" of the mature fruit. Wherever stomata are formed in the epidermis, the hypodermal cells are interrupted by a substomatal air chamber (Figures 3, 5, 7). Between hypodermis and the main parenchyma of the flesh, about 15 layers (sub-hypodermis) also remain small-celled *(14, 15)* and experience active divisions until late in development (about 12 weeks after flowering). Hence the cells here have accommodated to the tremendous tangential expansion of the fruit periphery by cell division rather than cell extension. Tangential expansion occurs late and is relatively minor. These cells acquire thickened walls like those of the hypodermis about 10-11 weeks after flowering (Figures 10, 12, 13). Hence there is often a distinct boundary between the skin and flesh regions in the mature fruit.

"Flesh." During the formation of the bulk of the fruit flesh, the parenchyma cells actively divide and enlarge. Adjacent to the ovary, divisions are principally tangential, and the main direction of cell enlargement here is radial (Figure 2). Mitchell *(14)* has given the average diameters of these more interior cells at harvest as 120 μ (radial diameter) $\times$ 82 μ (tangential diameter). Farther out from the ovary, up to the skin, cell divisions have no constant orientation, and cell enlargement is more isodiametric (Figure 3). Mitchell's *(14)* figures for cells in this region are 185 μ (radial diameter) $\times$ 165 μ (tangential diameter). Together, these two zones comprise about 250-cell layers at harvest, indicating almost a ten-fold increase in cell number radially. The

---

All figures are cross sections of fruit of Bartlett pear. Ovary is located toward base of page in each figure. All $\times$ 92.

Figure 7. Portion of surface of large fruit at 4 weeks. Stomatal opening is becoming wider. Sclereids beginning to differentiate in hypodermis as well as sub-hypodermal region.

Figure 8. Portion of inner flesh at 6 weeks. Sclereids still being initiated from nearby cells of radiating rows of parenchyma.

Figure 9. Portion of inner flesh at 10 weeks. Note conspicuous enlargement of cells as compared with figure 8. Radial enlargement most pronounced in cells radiating from cluster. No more cell divisions or new sclereid differentiation occurring here.

Figure 10. Portion of fruit surface at 10 weeks. Contrast cell size with that of figure 9. Cell divisions and sclereid initiation are both active here. At upper left is lenticel region unrelated to stoma.

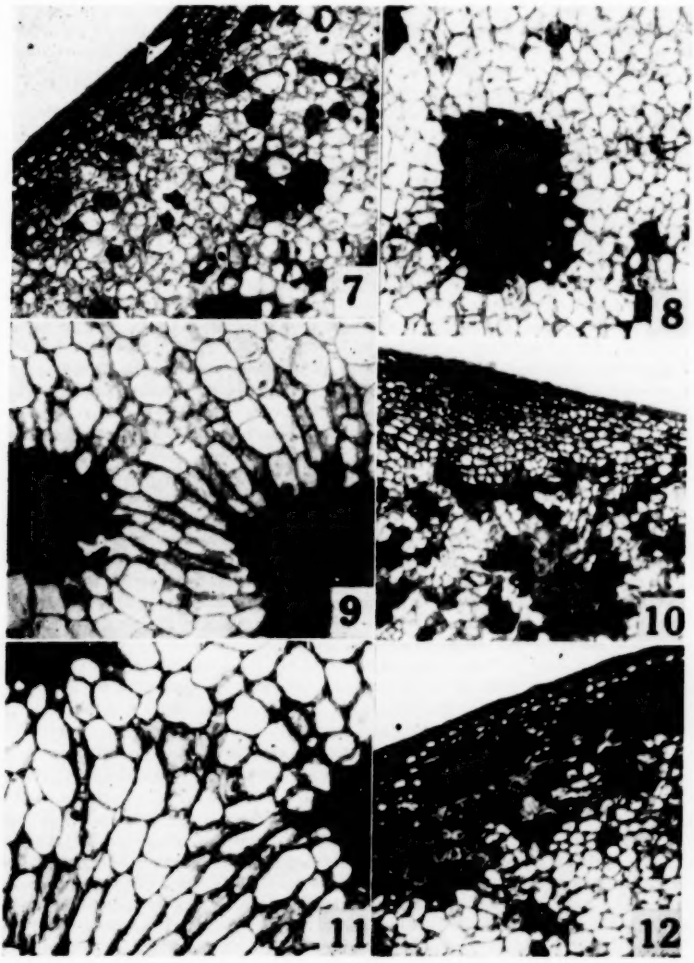
Figure 11. Portion of inner flesh at 12 weeks. Starch grains of parenchyma cells are small and lie in parietal cytoplasm. Enlargement of parenchyma cells is continuing slowly. A sclereid cluster is located just out of photomicrograph at lower left.

Figure 12. Portion of outer flesh at 12 weeks. Divisions ceasing in hypodermis and subhypodermis, with cell wall thickening becoming pronounced in both. Some sclereids still being initiated in radial direction at inner edges of sclereid clusters (see cluster at left).

major increase in production of new cells here occurs during the first 6-8 weeks after flowering. A characteristic feature of this parenchymatous region is the presence of numerous air spaces at the cell interstices. These first appear 6 weeks after flowering (Figure 8) and gradually increase in number and size as development progresses.

Sclereid differentiation. Sclereids first differentiate among the parenchyma cells about two weeks after flowering (Figures 2, 3). In larger fruits obtained at this time, isolated cells have already acquired slightly thickened walls which are in the process of becoming lignified. No pattern of cellular differentiation seems clear among these first-differentiated sclereids. They appear at random among their neighbors, often unevenly spaced from one another, some adjacent, others far apart. The first cells differentiate near the ovary and the subsequent ones progressively farther towards the periphery of the fruit. None ever occurs in the epidermis or in the ovarian tissues.

In the course of this differentiation, a parenchyma-like cell begins to enlarge and becomes distinct from its neighbors by its larger size. As it reaches full size, the cell



is found to be occupied by a large central vacuole surrounded by a parietal layer of cytoplasm. After growth in cell diameter has ceased, the wall begins to thicken and thus demonstrates the presence of numerous simple pits over its surface. Lignification and thickening of the cell wall continue further, leaving laminations in the cell wall as the lumen, or cell interior, becomes progressively smaller.

Hand in hand with the apposition of new layers of cell wall material goes the prolongation of the pits into long, tubular canals. As the area of interior wall becomes successively smaller, some of these canals run together to form a single one; this in turn may coalesce with another pit canal farther to the interior as the wall thickens. Thus the mature sclereid often shows a "ramiform" or branched type of pitting in its cell wall. At the middle lamella, pits of adjoining sclereids are distinctly lined up with each other; none end blindly against an unpitted portion of wall of a neighboring cell.

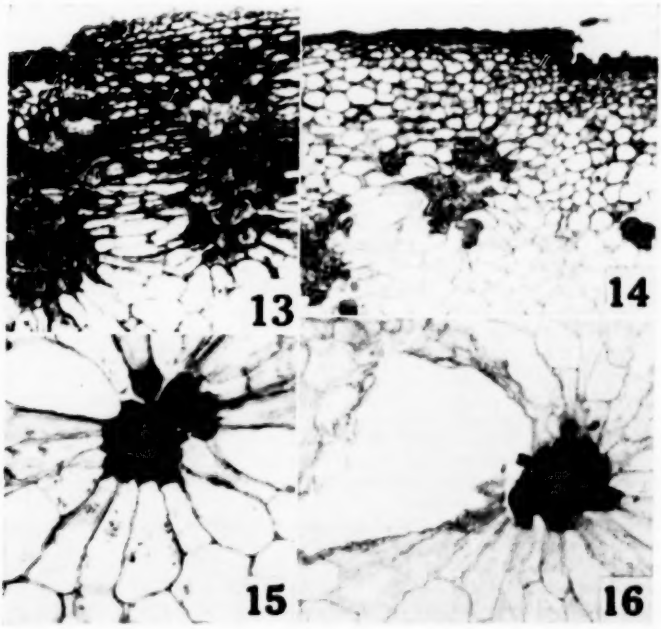
The mature sclereid is composed almost entirely of the cell wall. The lumen is reduced to a small spherical or linear space in the center of the cell, and the bulk of the cell volume is made up of the centripetally deposited, laminated cell wall. The wall is composed principally of cellulose and lignin, the latter being indicated by the phloroglucinol-HCl reaction. During its maturation, the cell remains uninucleate, and its cytoplasm becomes increasingly granular in appearance. Whether the cell remains alive for any length of time after the program of wall thickening is completed could not be determined from the fixed material used here.

Formation of sclereid clusters. Although the first-formed sclereids in the young fruit initially are larger than the cells of the adjoining parenchymatous tissue, later-formed sclereids may be scarcely different in size from their neighbors and may even be smaller in some cases, during rapid enlargement of the parenchyma cells. The later-formed sclereids may occur isolated from other sclereids, as "idioblasts," or more frequently they may differentiate from parenchymatous cells adjoining a previously-formed sclereid (Figures 4, 5). In the latter case, there is no constant positional relationship between the older and the younger sclereid. They may be side by side, radially, vertically, tangentially, or obliquely, with the first two relationships encountered somewhat more frequently than the last two. They may or may not be daughter cells of a single mother cell (radially and vertically adjoining sclereids generally have been derived from the division of a mother cell). In these later-differentiated cells, the course of differentiation follows the program of the first-formed ones. Often a complete sphere of new sclereids will develop about the first one as a nucleus of differentiation (Figure 4).

As the sclereid cluster is thus initiated, surrounding parenchymatous cells reflect the organization of that cluster by the orientation of their cell divisions. While the cluster is small (3-4 cells in diameter), divisions in the adjoining cells are now oriented so that the new walls are formed in a direction concentrically about or tangentially to the sclereid cluster (Figures 2, 4). These divisions are repeated sequentially in the daughter cells produced by the first such divisions. Thus there soon appear rows of parenchymatous cells which radiate on all sides from the small sclereid cluster (Figures 6, 8). Since the sclereids as such do not divide, further cluster growth must be at the expense of these radially oriented parenchymatous cells, and this development is reflected in the arrangement of the outer cells of the sclereid cluster.

Although initially the sclereid clusters may be separated by only 1-3 parenchymatous cells, divisions in these intervening cells can be so numerous as to permit each sclereid cluster to gain 4-5 cell layers radially and still be separated by 5-10 layers of undifferentiated parenchyma cells (Figures 9, 11). Near the surface of the fruit, where sclereids are initiated later and continue to be produced longer than in the interior, more isolated sclereids are found at maturity, and the clusters tend to be smaller (Figures 5, 7, 10, 12). The largest clusters and densest concentration of clusters occur just outside the ovary *(16, 17, 27)*.

Usually, wherever formed, the development of the cluster from surrounding cells follows a pattern similar to that described above. However, near the periphery of the fruit, there are two tendencies in sclereid development which do not appear elsewhere: (a) of the very few sclereids which develop from cells furnished by the original hypodermal layer, most are isolated idioblasts; and (b) there is a strong tendency for the sclereid cluster formed from the small-celled sub-hypodermal parenchyma to extend in a radial direction and thus become more prismatic than spherical (Figures 12, 13).



All figures are cross sections of fruit of Bartlett pear. Ovary is located toward base of page in each figure. All × 92.

Figure 13. Portion of surface of fruit at 16 weeks. Torn stoma at upper left. Irregular epidermis at upper right. Note radial organization of sclereid clusters, heavy thickening of collenchymatous cells of skin, and elongation of cells laterally and internally about sclereid clusters.

Figure 14. Portion of surface of fruit at 20 weeks. Torn stoma at upper right. Irregular callus-like cellular protuberances are visible at base of stoma. Thickening of collenchymatous cells is mostly gone, and cell walls of parenchyma of flesh are deteriorating.

Figure 15. Portion of flesh of fruit at 16 weeks. Starch grains are readily visible in large parenchyma cells radiating from sclereid cluster.

Figure 16. Portion of flesh of fruit at 20 weeks. Starch has disappeared. The cell walls of the parenchyma have become thinner and stain less intensely. Some cells have broken down, with the formation of a cavity in the flesh. Note that sclereids are unaltered.

Tissue Maturation

The formation of new sclereids in the interior of the fruit flesh comes to an end about 8 weeks after flowering. Those sclereids which have been formed acquire thicker walls, and cell division ceases in the parenchymatous matrix about the sclereids. These surrounding parenchyma cells begin to enlarge rapidly, acquiring very large vacuoles in the process. Many begin to accumulate starch grains of both simple and compound (up to four moieties per grain) structure.

Although the increase in cell size principally represents an equivalent enlargement of the original cell shape, in the vicinity of the sclereids cell enlargement follows a different program. Here, the surrounding radiating rows of cells elongate radially from the cluster (Figures 9, 11, 15). Where two clusters are fairly close together, the rows of cells resemble the lines of force between two magnets. However, such resemblance is purely superficial, and often there will be less radial enlargement on the side

toward a relatively close cluster than on the side toward a more distant one (Figure 11). Turpin *(37)* described the flesh of pear as composed of flower-like units, each consisting of a stony "nucleus" and radiating, elongated cells.

At the periphery of the fruit, parenchymatous cell division and sclereid initiation continue up to 12 weeks after flowering (Figures 10, 12). Hence the sub-hypodermal parenchymatous cells are relatively small and the sclereid clusters relatively close together during rapid growth of the fruit. Even at 12 weeks, new sclereids are being added in a radial direction at the inner edges of sclereid clusters in the sub-hypodermis (Figure 12). As cell divisions cease, the parenchymatous cells on the interior side of the sclereid clusters as well as those lying tangentially alongside the clusters begin to extend radially from the cluster as a center (Figure 13). However, the clusters still are much closer together than those in the interior flesh, giving a relatively high concentration of sclereids in the skin. On the outer side of the cluster, the amount of cell elongation varies directly with the distance of these cells from the hypodermis itself. The collenchyma cells of the hypodermis and the few adjoining layers of parenchyma cells enlarge tangentially.

Fruit Ripening

Despite separation of sclereid clusters by the continuing enlargement of the intervening cells, the fruit remains relatively firm and hard, and the flesh preserves its gritty texture to within two weeks before fruit abscission. In the succeeding two weeks, the fruit becomes soft, tender and succulent, and the grit is scarcely perceived when the flesh is chewed.

Microscopically, the principal changes occurring during this two-week interval are concerned with the parenchyma cells of the flesh and the collenchyma cells of the skin. The latter begin to lose most of their wall thickening and come to resemble ordinary parenchyma cells (Figure 14). In the parenchymatous matrix of the flesh, starch disappears, the cell walls become extremely thin and tenuous, and in some areas there may be cellular separation and collapse (Figure 16) as in prune *(23)*. No changes are evident in the number of sclereid cells per cluster, in the distribution of the clusters *(15)*, or in the structure of the individual sclereids.

After being stored for one additional week at 70°F., the pears soften considerably and are almost past the optimum ripe eating stage. Microscopic examination of specimens from these fruits indicates extreme thinning of the walls of the collenchyma cells of the skin and further wall thinning and cellular collapse in the parenchyma of the flesh. Sclereids are unaltered in number, structure, and distribution.

DISCUSSION

The results of a microscopic study of pear development show that degradative changes in the sclereids themselves do not occur in the course of maturation. There is no evidence of wall-thinning or delignification in the walls of these cells. Softening of tissues in the flesh of the Bartlett pear results from a breakdown in the walls of the parenchyma cells. The sclereid clusters thereby are embedded in a soft matrix, which permits chewing of the flesh without crushing sclereid groups when the teeth are brought together. The further topographical separation of sclereid clusters in the fruit during the last stages of ripening also contributes to this organoleptic effect.

However, in two varieties of the quince, which is closely related to pear, Alexandrov and Djaparidze *(2)* noted a definite decrease in the lignin content of the sclereids, accompanied by thinning of the cell wall during storage of the fruit. Alexandrov and Alexandrova *(1)* cited several investigations and conducted their own studies to indicate resorption of the lignified walls of tracheary elements in older parts of the plant. On bending stems of flax and hemp plants, Schilling *(19, 20)* was able to produce

localized instances of delignification in fiber cells. Bloch *(4)* has reviewed literature dealing with the phenomena of delignification as a result of traumatic stimuli. On the other hand, some authors *(10, 29)* have rejected the possibility of autonomous delignification.

The chemical evidence of Crist and Batjer *(8)*, that the ligno-cellulose content decreases during late maturation, is based on percentage dry weight changes in the pear flesh. Since other materials (starch, sugars, pectic substances, etc.) can and do change in amount during development, it is obvious that percentage changes on a fixed weight basis may be misleading. Thus, lignocellulose content may *appear* to decrease if other solid constituents increase in the cells of the fruit. That such is the case in pear, may be seen in the study of Smith *(21)*. Smith duplicated Crist and Batjer's procedure and obtained the same results. However, he also analyzed whole fruits and showed an entirely different picture of development, namely, a small, constant increase in the amount of cellulose and lignin and a much greater increase in other constituents, such as reducing material and other alcohol-soluble compounds. Hence there is no evidence for delignification of the sclereids of pear during maturation.

It is of theoretical interest to relate the initiation and formation of sclereid clusters in pear to other studies dealing with differentiation in plant tissues. Since the first sclereids appear truly at random, *i.e.*, without constant spacing from each other, their formation cannot be due to using up all the available substrate in their vicinity, as postulated by Wigglesworth *(28)* for bristle-plaque formation in *Rhodnius*. Nor can it involve the release of inhibitory substances from each cell *(6)*, which would also insure even spacing of sclereid initials and prevent side-by-side sclereid differentiation. Rather it appears that a "homoiogenetic induction" *(5)* is promoted, so that adjoining cells are stimulated to become sclereid initials. The production of concentrically oriented cell divisions in surrounding cells is further evidence of this influence. At this point, it should be remarked that there is no support for Cave's *(7)* view that a sclereid cluster is derived from divisions in a single mother cell.

Like sclereid development in Douglas fir *(22)*, initiation of a sclereid in pear is followed by a continuous, uninterrupted program of development, including extensive wall-thickening and lignification. No cases of partial development occur. However, unlike sclereid development in Douglas fir, sclereid formation and growth in pear is not related to the formation of intercellular air spaces. A discussion of the relationship between sclereid development and air spaces has been presented earlier *(22)*.

SUMMARY

Sclereid development in the Bartlett pear was studied microscopically in order to determine the role of sclereids in the texture of the mature pear. Sclereid clusters are formed in both flesh and skin, being relatively more numerous in the latter and just outside the ovary.

Initiation of sclereid formation takes place at random throughout the pear flash. Usually the sclereid initial is a nucleus for formation of new sclereids from adjoining parenchymatous cells. Serial concentric divisions in parenchyma cells surrounding the small sclereid cluster produce radiat-

ing files of cells, from which new sclereids differentiate at the surface of the sclereid cluster.

After complete maturity of the sclereid (extensive wall-thickening and lignification), no further changes occur in its structure or composition. The softening of the mature fruit is due not to delignification of the sclereids but to degradative changes in the cell walls and collapse of the intervening parenchyma cells.

LITERATURE CITED

1. Alexandrov, W. G., and Alexandrova, O. G. Ist die Verholzung ein reversibler oder irreversibler Vorgang? *Planta*, **7**, 340-346 (1929).
2. Alexandrov, W. G., and Djaparidze, L. I. Über das Entholzen und Verholzen der Zellhaut. *Planta*, **4**, 467-475 (1927).
3. Bell, H. P. The protective layers of the apple. *Canadian J. Res.*, **15C**, 391-402 (1937).
4. Bloch, R. Wound healing in higher plants. *Bot Rev.*, **7**, 110-146 (1941).
5. Bünning, E. Entwicklungs und Bewegungsphysiologie der Pflanze. *Lehrb. Pflanzenphysiol.*, **2**, 3. 1948, Springer Verl. Berlin.
6. Bünning, E., and Sagromsky, H. Die Bildung des Spaltöffnungsmusters in der Blattepidermis. *Zeitschr. Naturf.*, **3b**, 203-216 (1948).
7. Cave, C. Structure et développement du fruit. *Ann. Sci. Nat. Bot.*, V **10**, 123-190 (1869).
8. Crist, J. W., and Batjer, L. P. The stone cells of pear fruits, especially the Kieffer pear. Michigan Agricultural Experiment Station *Tech. Bull.* **113** (1931).
9. Douglas, G. E. The inferior ovary. *Bot. Rev.*, **10**, 125-186 (1944).
10. Frey, A. Über die Intermicellar-Räume der Zellmembranen. *Ber. D. Bot. Ges.*, **46**, 444-456 (1928).
11. Johansen, D. A. *Plant Microtechnique*. 1940, McGraw-Hill Book Co., Inc., N. Y.
12. Lilleland, O. Growth study of the apricot fruit. *Proc. Amer. Soc. Hort. Sci.*, **27**, 237-245 (1930).
13. Malfatti, J. Beiträge zur Anatomie der Birn und Apfelfrucht. *Zeitschr. Nahrungsmittel-Unters. Hyg.*, **10**, 265-269 (1896).
14. Mitchell, A. E. Gross morphology and histology of the developing fruit of the Bartlett pear. (Ph.D. thesis, The Ohio State Univ., 1950).
15. Murneek, A. E. Studies of physical and morphological changes in Bartlett pears. *Am. J. Botany*, **10**, 310-324 (1923).
16. Osterwalder, A. Blütenbiologie, Embryologie und Entwicklung der Frucht unserer Kernobstbäume. *Landwirtsch. Jahrb.*, **39**, 917-998 (1910).
17. Potonié, H. Über die Bedeutung der Steinkörper im Fruchtfleische der Birnen und der Pomazeen u. überhaupt. *Kosmos*, **8**, 33-36 (1880).
18. Ragland, C. H. The development of the peach fruit, with special reference to split-pit and gumming. *Proc. Amer. Soc. Hort. Sci.*, **31**, 1-21 (1934).
19. Schilling, E. Über die lokalen Anschwellungen der Bastfasern. *Ber. D. Bot. Ges.*, **39**, 379-383 (1921).
20. Schilling, E. Ein Beitrag zur Physiologie der Verholzung und des Wundreizes. *Jahrb. Wiss. Bot.*, **62**, 528-562 (1923).
21. Smith, W. W. The course of stone cell formation in pear fruits. *Plant Physiol.*, **10**, 587-611 (1935).
22. Sterling, C. Sclereid formation in the shoot of *Pseudotsuga taxifolia*. *Am. J. Botany*, **34**, 45-52 (1947).
23. Sterling, C. Developmental anatomy of the fruit of *Prunus domestica* L. *Bull. Torrey Bot. Club*, **80**, 457-477 (1953).
24. Tetley, U. A study of the anatomical development of the apple and some observations on the "pectic constituents" of the cell-walls. *J. Pomol. Hort. Sci.*, **8**, 153-172 (1930).
25. Tetley, U. The morphology and cytology of the apple fruit, with special reference to the Bramley's Seedling variety. *J. Pomol. Hort. Sci.*, **9**, 278-297 (1931).

26. TUKEY, H. B., AND YOUNG, J. D. Histological study of the developing fruit of the sour cherry. *Bot. Gaz.*, **100**, 723-749 (1939).

27. TURPIN, P. J. F. Mémoire sur la différence qu'offrent les tissus cellulaires de la Pomme et de la Poire; sur la formation des concrétions ligneuses de la dernière, celle des noyaux et du bois, comparées aux concrétions calcaires qui se trouvent sous le manteau des Arions, et à l'ossification des animaux en général. *C. R. Acad. Sci., Paris*, **6**, 711-738 (1838).

28. WIGGLESWORTH, V. B. The role of the cell in determination. *Soc. Exp. Biol. Symp.*, **II**, 1-16 (1948).

29. WISSELINGH, C. VAN. Die Zellmembran. *Handb. d. Pflanzenanat.* **III**/2. 1925, Gebr. Borntraeger. Berlin.

30. ZSCHOKKE, A. Ueber den Bau der Haut und die Ursachen der verschiedenen Haltbarkeit unserer Kernobstfrüchte. *Landwirtsch. Jahrb. Schweiz*, **11**, 153-197 (1897).

LYSINE DESTRUCTION IN CASEIN-GLUCOSE INTERACTION MEASURED BY QUANTITATIVE PAPER CHROMATOGRAPHY*

A. R. PATTON, R. C. SALANDER,[b] AND MARCUS PIANO
Chemistry Section, Colorado Agricultural Experiment Station, Fort Collins, Colorado

(Manuscript received February 5, 1954)

The reaction or reactions between proteins and carbohydrates involving non-enzymatic browning or discoloration of foods during processing and storage has been referred to as the Maillard reaction, protein-sugar reaction, or carbonyl-amino reaction (reviewed by Hodge, *5*). A decrease in nutritive value resulting from such reactions has been attributed chiefly to creation of lysine deficiency (reviewed by Patton, *10*). Under certain circumstances it has been found that a portion of the lysine is not recoverable by acid hydrolysis of the treated protein, and this portion is said to have been "destroyed." However, evidence for lysine destruction in proteins must be regarded as circumstantial, because the "destroyed" lysine has never been measured directly. Moreover, the determination of the remaining lysine in such an environment has presented a difficult analytical problem. Microbiologic assay, the principal method hitherto employed, is particularly objectionable, because it involves additional browning reaction when the hydrolysate is autoclaved with glucose prior to inoculation, and because it is subject to errors of specificity.

The present work resulted from a search for a new and better analytical tool for measuring such amino acid destruction. This approach began in 1948 when Patton, Hill, and Foreman *(13)* chromatographed each of the dietary-essential amino acids, before and after heating with glucose, and observed that lesser amounts of certain of the amino acids remained after browning. This was followed by several years of research on the methodology of paper migration analysis *(12, 14)*, resulting in modifications of the maximal density method of Block *(1)*, and of the area method of Bull, Hahn, and Baptist *(2)*. The authors are not aware of any publication in which a similar application of quantitative paper chromatography has been reported.

Certain observations of Lea and co-workers (reviewed by Hodge, *5*) are especially pertinent to the present report. They found no destruction of lysine from acid hydrolysis in the presence of added glucose. At 37°C. (98.6°F.), they found the most rapid and extensive reaction to occur in a state of incipient dryness *(6)*. By use of the Sanger technique *(15)*, they demonstrated the principal points of attack on the lysine residues to be the epsilon-amino groups, and a small amount of alpha-amino groups contained on a few terminal lysyl residues *(16)*.

*Published with the approval of the director, Colorado Agricultural Experiment Station, as Scientific Series Paper 430. Supported in part by a grant from The Nutrition Foundation.

[b]Present address, Dept. of Agricultural Biochemistry, Rutgers University.

EXPERIMENTAL PROCEDURE

Free amino acids were studied by dissolving 100 mg. of each chromatographically-pure amino acid in 25 ml. of 5% chromatographically-pure anhydrous D-glucose solution. From this volume, a 10-ml. aliquot was transferred to a small beaker, and evaporated to a concentrated syrup on a water bath at 90°C.(194°F.). Each sample was then autoclaved 2 hrs. (moist heat). The resulting samples were then diluted with water to 10 ml. at room temperature, and chromatographed alongside the unbrowned aliquots to obtain comparative increment-curve areas for both the amino acid and glucose *(2)*.

Casein was subjected to a number of different treatments, chosen on a basis of experience in preliminary trials. Each treatment was applied to 10 replicate samples, hereafter referred to as "techniques." Each technique contained a mixture of 1.0000 g. Difco isoelectric casein and 1.000 g. chromatographically-pure anhydrous D-glucose. Ten replicate spots were chromatographed from each technique, yielding 10 values for lysine content. Thus the per cent lysine remaining in a hydrolysate after each treatment was calculated from 100 values as determined by paper chromatography.

Humidity equilibration, where used, was accomplished by storing the techniques 48 hrs. in an atmosphere of 70% relative humidity, obtained over sulfuric acid sp. gr. 1.25 in a closed system. All autoclaving was done at 120°C.(248°F.). Heat treatment consisted of autoclaving 4 hrs. Acid hydrolysis consisted of autoclaving 6 hrs. with 100 ml. 6N hydrochloric acid. During both heat treatment (with one exception) and acid hydrolysis, the techniques were sealed in 12-oz. "pop" bottles, using dental dam under the cork gaskets of the crown caps.

Enzyme digestion consisted of incubating in 100 ml. of 1% trypsin at pH 7.8 and 37.5°C.(99.5°F.) for 10.5 hrs. Dinitrophenyl derivatives were prepared by the method of Sanger *(15)*. Reference compounds were prepared from lysine and from alpha-N-acetyl lysine.[c] In the latter case, treatment with 2,4-dinitrofluorobenzene (DFB) formed the epsilon-2,4-dinitrophenyl (DNP) derivative, after which the acetyl groups were detached by hydrolysis.

The general methodology of amino acid analysis by quantitative paper chromatography as employed in this laboratory has been previously described *(11, 12, 14)*. In the present work, 1-μl. aliquots of the properly-diluted hydrolysates were chromatographed by the ascending method on 17 × 22 inch sheets of Whatman No. 1 paper in water-saturated 2,4-lutidine (Matheson). Time of chromatography ranged from 18 to 24 hrs. All operations were conducted at room temperature, controlled at approximately 22°C. (71.6°F.). For the lysine values within each technique, peak density was measured with a densitometer and compared with the standard lysine curve obtained from reference lysine spots on the same paper. Density curve areas were obtained by plotting the density readings against Rf in 3 mm. increments, and measuring the areas enclosed by the curves with a polar planimeter. The glucose spots were developed by spraying with aniline-hydrogen-phthalate solution and heating *(9)*, a method found by experiment to yield quantitative results for D-glucose by either the area or maximal color density method. Statistical analyses (calculations by R.C.S.) were conducted according to the methods of Villars *(17)*.

RESULTS AND DISCUSSION

Although all of the commonly-occurring amino acids were browned with glucose and chromatographed for comparative destruction studies, only pertinent observations with regard to lysine will be reported here. DL-lysine·HCl·H_2O, when heated in the absence of glucose, underwent no demonstrable change. Identical quantities in identical spots were obtained before and after heating whether chromatographed in *n*-butanol-water-acetic acid *(9)*, or in water-saturated lutidine. However, after heating with glucose, less lysine appeared at the original Rf and a new ninhydrin-reacting spot appeared at a higher Rf. Specificity of chromatographic detection

[c] The DL-lysine and alpha-N-acetyl lysine were kindly provided by Dr. J. Waddell, E. I. duPont de Nemours & Co., Wilmington, Delaware.

is based on both ninhydrin reaction and Rf. Here a lysine-glucose intermediate would not be mistaken for lysine even though it reacted with ninhydrin, because the Rf would be different. Such an intermediate was isolated by Chichester from a glucose-glycine system *(5)*. Although it seems likely that this higher-Rf material was such an intermediate, attempts to isolate it by paper chromatography were unsuccessful. Upon rechromatographing such eluates, only spots at the original lysine Rf appeared.

Free lysine and glucose were also heated together in the presence of each of 25 other amino acids. From this series it was observed that when free tryptophan was present, it appeared to spare lysine. So long as any tryptophan remained, practically no destruction of lysine occurred. At the same time the tryptophan suffered extensive, and in some cases complete, destruction as indicated by loss of ninhydrin-reacting capacity.

When D-glucose was heated air-dry in an oven at 150°C.(302°F.), and successive aliquots were chromatographed and treated with the aniline-hydrogen-phthalate reagent, there was a steady decline in spot density. At 24 hrs. the reaction was negative, suggesting that all the glucose had been destroyed. Glucose decomposition with browning occurred under much milder conditions in the presence of an amino acid, indicating that the browning reaction may be considered to be amino-catalyzed. Evidence has been published that, although the final reaction involves a liberation of carbon dioxide from glycine, in an intermediate reaction glucose is degraded and glycine remains unchanged *(5)*. In the present work, it was found possible to obtain complete "destruction" of glucose as indicated by a negative reaction to the aniline-hydrogen-phthalate reagent on chromatograms, with practically no loss of glycine. In the presence of excess of a sulfhydryl compound, such as cysteine, it was also shown that all of the glucose could be destroyed, and most of the amino acid retained, without much brown color formation. The findings reported in this paragraph are in agreement with the observations of others *(5)*; it is believed, however, that they are much more easily demonstrated by paper chromatography than by the methods more customarily employed.

The percentages of lysine remaining in casein as released by trypsin digestion were as follows: casein untreated, 6.0; casein autoclaved without glucose, 5.2; casein-glucose mixture untreated, 3.2; and casein-glucose mixture autoclaved, 1.2. The low value of lysine in the untreated casein resulted from the incomplete hydrolysis one expects from enzyme digestion. The decrease in lysine obtained from the autoclaved casein is evidence of the so-called "heat damage" reaction, which has been postulated to be a formation of enzyme-resistant linkages through interaction of the epsilon-amino groups of lysine residues with the free carboxyl groups of aspartic and glutamic acid residues *(10)*. The decrease in the unheated casein-glucose mixture was probably due to interaction during storage before digestion, as found by Lea and Hannan *(6)*. The value for the autoclaved casein-glucose mixture was probably the combined result of lysine destruction plus formation of enzyme-resistant linkages through the carbonyl-amino reaction *(10)*. These results confirm those reported by others *(3, 4, 7, 8)*.

The percentages of lysine remaining in the hydrolysates from the main series of treatments were as follows: (A) casein, equilibrated and autoclaved, 7.4367 ± 0.73 (standard deviation of 100 values); (B) casein, untreated, 7.7084 ± 1.45; (C) casein and glucose, equilibrated and autoclaved, 6.9171 ± 1.00; (D) casein and glucose, untreated, 7.6260 ± 1.10; (E) casein plus 100 ml. water, autoclaved, 7.3137 ± 1.22; (F) casein and glucose plus 100 ml. water, autoclaved, 5.0014 ± 0.95; (G) casein and glucose, sealed and incubated 30 days at 37°C., 7.0076 ± 1.49; and (H) casein and glucose, autoclaved uncapped to allow introduction of steam from the autoclave, 1.6100 ± 0.22. Inasmuch as the lysine values shown were intended for comparative purposes only, they are uncorrected for moisture, ash, etc., in the original casein sample used throughout. The values shown here and in Table 1 were carried to four decimal places for purposes of calculation only, and are not intended to imply that the authors believe the analytical method to be accurate to this extent.

TABLE 1

Effect of various treatments on lysine content of casein

Treatments compared	Difference (% lysine)	Least significant difference		Standard deviation
		0.05	0.01	
A and B	0.2717	0.3172	0.4160	1.140
A and C	0.5196	0.2448	0.3217	0.883
A and D	0.1893	0.2600	0.3420	0.940
C and D	0.7089	0.2920	0.3830	1.05
A and E	0.1230	0.2700	0.3550	0.980
A and F	2.4353	0.2340	0.3070	0.844
D and F	2.6246	0.2850	0.3750	1.03
E and F	2.3123	0.2960	0.3890	1.07
A and G	0.4291	0.3330	0.4380	1.14
A and H	5.8267	0.3119	0.2910	0.382

Data in Table 1 were obtained from variance analyses not shown. In this table comparison of treatment (A) with treatment (B) shows that there was no significant destruction of lysine due to heating casein alone. Comparison AD indicates that there was no significant destruction of lysine due to acid hydrolysis in the presence of 1% glucose. However, in this case a difference was observed in total increment curve areas which was significant at the 0.01 level, indicating significant destruction of amino acids other than lysine during the hydrolysis due to the presence of glucose. The total area value for treatment (A) was 101.16 ± 6.79; for treatment (D), 97.00 ± 5.73. This represents a difference in area of 4.16, whereas variance analysis showed a difference of 2.29 to be significant at the 0.01 level. Similar area measurements were made for the other treatments, but the figures are not presented here, because they paralleled the lysine values to a marked degree. No destruction of tryptophan or cystine could be measured, because paper chromatograms indicated that these amino acids were destroyed during the acid hydrolysis with or without added glucose. Furthermore, in exploratory trials with higher proportions of glucose, hydrolysis in the presence of 2 g. decreased

the lysine per cent by 0.4, and in the presence of 4 g. by 1.7, suggesting that in the presence of glucose in a greater than 1:1 ratio the lysine in casein may undergo some destruction during acid hydrolysis. Comparison AE shows that no significant destruction of amino acids occurred due to heating casein in the presence of excess water.

A second set of comparisons shows differences, all of which were statistically significant at the 0.01 level; comparison AC showed significant, although relatively slight, destruction of lysine during autoclaving of the humidity-equilibrated casein-glucose mixture. Greater destruction of lysine occurred from autoclaving in 1% glucose solution, as shown by comparison AF. The greatest destruction of lysine occurred when the mixture of casein and glucose was autoclaved with free access to moisture from the steam of the autoclave, as shown in comparison AH. Comparison CD indicates that the lysine destruction occurred during the initial 4 hrs. of autoclaving in the presence of glucose and not during the subsequent hydrolysis. This is shown again by comparison DF. Comparison EF shows that lysine destruction was due to the presence of the glucose and not the water *per se*. Comparison AG shows that slight destruction of lysine occurred during storage at a warm temperature in a state of incipient dryness, and this difference was significant only at the 0.05 level.

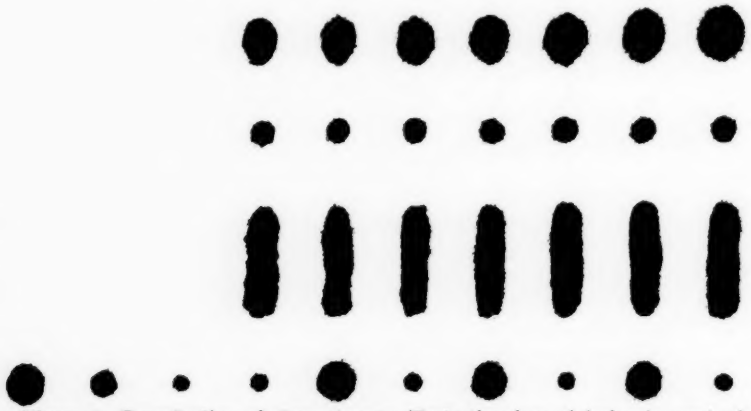


Figure 1. Reproduction of chromatogram illustrating loss of lysine in casein due to carbonyl-amino reaction.

Figure 1 shows a Contoura reproduction of a chromatogram prepared to show destruction of lysine in casein. It contains, from left to right, three spots in decreasing concentration for the lysine standard series, followed by parallel rows of spots obtained from alternate applications of hydrolysates from treatments H and A respectively. The differences in size of the lysine spots (bottom row) clearly reveal the decrease in lysine concentration due to destruction by the carbonyl-amino reaction. The extreme contrast of the reproduction process has eliminated the density differences on which the quantitative measurements would be based, as well as a string of weak spots, including the spot for arginine, which occurrd in the

blank space above lysine. The spots above lysine constitute the remainder of the amino acid spectrum for casein, minus tryptophan and cystine. It should be pointed out that quantitative paper chromatography is not limited to the determination of lysine. Solvent systems can be chosen which permit estimation of any of the amino acids. Here no attempt was made to achieve better separation than was necessary for the isolation and estimation of lysine. The identity of the lysine spot was established by recovery analyses on a casein hydrolysate to which known quantities of lysine had been added, and by the disappearance of this spot when casein was treated with DFB.

The treatment of casein with DFB to form the DNP derivatives on free amino groups resulted in the virtual disappearance of the casein-lysine *per se*, and the appearance instead of a new yellow spot on chromatograms, consisting of the epsilon-DNP derivative of lysine. A second yellow spot, very small, was probably due to di-DNP derivative of lysine, due to its presence to a certain extent in the terminal position. When casein from treatment H, containing only 1.610% lysine, was treated with DFB, the excess removed, the residue hydrolyzed, and chromatographed, the yellow spots representing the presence of DNP-lysine were virtually absent, providing evidence that the free amino groups on the lysine residues had been blocked by the carbonyl-amino reaction. After this experiment was completed, Schwartz and Lea *(16)* published similar results.

SUMMARY

Objectives of this investigation were to evaluate the precision of the quantitative paper chromatography method, to establish the destruction of lysine in casein with greater certainty, and to measure the extent of such destruction due to the carbonyl-amino reaction under selected conditions of treatment. Under the conditions employed, the most severe loss of lysine occurred from autoclaving with moist heat. During four hours of autoclaving, only minor destruction occurred in the relative absence of moisture or in the presence of an abundance of moisture. Without glucose no significant destruction occurred. The precision of the analytical method was sufficient that differences in per cent lysine of approximately 0.36 were significant at the 0.01 level.

LITERATURE CITED

1. Block, R. J. Estimation of amino acids and amines on paper chromatograms. *Anal. Chem.*, **22**, 1327 (1950).

2. Bull, H. B,. Hahn, J. W., and Baptist, V. H. Filter paper chromatography. *J. Am. Chem. Soc.*, **71**, 550 (1949).

3. Cook, B. B., Fraenkel-Conrat, J., Singer, B., and Morgan, A. F. The effect of heat treatment on the nutritive value of milk proteins. III. The effect of heat on casein, lactalbumin, and their lactose-induced derivatives, with special reference to digestibility and rate of release of lysine, methionine, and tryptophan. *J. Nutrition*, **44**, 217 (1951).

4. Davis, R. M., Rizzo, P., and Smith, A. H. The effect of heat on the nutritive value of lactalbumin. *J. Nutrition*, **37**, 115 (1949).

5. Hodge, J. E. Chemistry of browning reactions in model systems. *J. Ag. Food Chem.*, **1**, 928 (1953).

6. LEA, C. H., AND HANNAN, R. S. Studies of the reactions between proteins and reducing sugars in the "dry" state. III. Nature of protein groups reacting. *Biochim. et Biophys. Acta*, **5**, 433 (1950).

7. LOWRY, J. R., AND THIESSEN, R. Studies of the nutritive impairment of proteins heated with carbohydrates. II. *In vitro* digestion studies. *Arch. Biochem.*, **25**, 148 (1950).

8. MCINROY, E. E., MURER, H. K., AND THIESSEN, R. The effect of autoclaving with dextrose on the nutritive value of casein. *Arch. Biochem.*, **20**, 256 (1949).

9. PATTON, A. R. Further student experiments with paper chromatography. *J. Chem. Educ.*, **27**, 574 (1950).

10. PATTON, A. R. Present status of heat-processing damage to protein foods. *Nutrition Revs.*, **8**, 193 (1950).

11. PATTON, A. R. Quantitative paper chromatography for students. *J. Chem. Educ.*, **28**, 629 (1951).

12. PATTON, A. R., AND CHISM, P. Quantitative paper chromatography of amino acids. *Anal. Chem.*, **23**, 1683 (1951).

13. PATTON, A. R., HILL, E. G., AND FOREMAN, E. M. The effect of browning on the essential amino acid content of soy globulin. *Science*, **108**, 659 (1948).

14. SALANDER, R. C., PIANO, M., AND PATTON, A. R. Accuracy of quantitative paper chromatography in amino acid analysis-addendum. *Anal. Chem.*, **25**, 1252 (1953).

15. SANGER, F. The free amino groups of insulin. *Biochem. J.*, **39**, 507 (1945).

16. SCHWARTZ, H. M., AND LEA, C. H. The reaction between proteins and reducing sugars in the "dry" state. Relative reactivity of the α- and ϵ-amino groups of insulin. *Biochem. J.*, **50**, 713 (1952).

17. VILLARS, D. A. *Statistical Design and Analysis of Experiments for Development Research*. 1951. William C. Brown Co., Dubuque, Iowa.

HEAT RESISTANCE OF STRAINS OF *SALMONELLA* IN LIQUID WHOLE EGG, EGG YOLK, AND EGG WHITE*

W. W. OSBORNE, R. P. STRAKA, AND HANS LINEWEAVER
Western Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Albany 6, California

(Manuscript received February 8, 1954)

Human and animal pathogens of the genus *Salmonella*, which occur frequently in foods, have been causes of concern to varying degrees for many years, as ably pointed out in the review article by Hinshaw and McNeil *(14)*. The potential health hazard has led naturally to studies of means for eliminating *Salmonella* from foods, including studies of heat-resistance *(4, 5, 9, 10, 11, 25, 27, 30, 31)*. The references cited concern liquid egg principally, since that is the subject of special interest in this report. These studies show that *Salmonella* organisms generally are killed at temperatures above 135°F. Several studies indicate that 1 to 4 minutes at 142°F. are required to "destroy" *Salmonella* in liquid whole egg *(11, 31)* when large numbers are present initially. However, Winter *et al. (31)* and Solowey *(25)* both noted the occurrence of heat-resistant strains. At 142°F.(61.1°C.) liquid egg begins to thicken and egg white coagulates in a short time. It is therefore important to determine and to use the minimum heat treatment that will provide adequate safety in the final product. Because of excessive coagulation on heating surfaces it is not practical ordinarily to use higher pasteurization temperatures [e.g. 160° to 180°F.(71.1 to 82.2°C.)] such as those used for dairy products *(7)*. Egg pasteurization is a borderline operation.

The purpose of this report is to extend our knowledge of the heat sensitivity of *Salmonella* as a basis for heat treatments or other steps that might be required to provide a satisfactory margin of safety for egg products of various types. Thermal death times and decimal reduction times are reported for a number of strains of *Salmonella* suspended in phosphate buffer and in whole egg, egg yolk, and egg white. Six of these strains were used by McCullough and Eisele *(18, 19, 20, 21)* in their studies of experimental salmonellosis. The effects of pH of suspending medium, numbers, and recovery method on the observed heat resistance will be described. It has not been possible in this study to evaluate certain other pertinent factors such as difference in heat resistance of young and old cells. Available information is discussed with respect to the limitations that exist for the pasteurization of egg products.

EXPERIMENTAL

Salmonella strains studied. The following strains, representing six types, were used: *S. derby* 5420 (type B), *S. barielly* CM 688 (type C_1), *S. newport* 5761 (type C_2), *S. pullorum* 3083 (type D), *S. anatis* 5343 and *S. meleagridis* 5485 (type E_1), and *S. senftenberg* 3252 and *S. senftenberg* 775W (type E_2). The numbers following

*Presented in part at the 1952 meeting of Institute of Food Technologists, Grand Rapids, Mich.

the species refer to strain numbers used at the Western Utilization Research Branch. Ten other strains or isolates were tested in a limited way.

Culture and test media. Trypticase soy agar and trypticase soy broth (BBL) were used as cultural media. Brilliant green agar (Difco) was used as the confirmatory medium.

The test media in which test organisms were suspended for heating consisted of liquid whole egg (prepared aseptically from fresh broken-out grade A shell eggs as needed) at pH 7.4-7.6 and whole egg acidified to pH 5.5; liquid egg white (from separated eggs) at pH 8.9-9.1 and the same acidified to pH 5.5; liquid egg yolk, pH 6.1-6.4; and M/15 phosphate buffer, pH 5.3, 7.0, and 8.2.

Preparation of bacterial suspension. A suspension of cells of a *Salmonella* culture was prepared by inoculating a trypticase soy agar surface in either Kolle flasks or Petri dishes with an actively growing culture of the organism. After 18 hours' incubation at 37°C. the growth was washed off with sterile 0.85% saline. The number of viable cells in such a suspension was usually about 4×10^{10}/ml. A suspension of organisms prepared in this manner remained stable when refrigerated at approximately 5°C. as indicated by viable count and constancy of heat resistance for more than 49 days.

Preparation of the whole egg melange. Fresh, grade A shell eggs were soaked in alcohol for 5 minutes, then removed, drained, and flamed to burn off the excess alcohol. They were then broken aseptically into egg cups with sterile equipment. After visual and olfactory examination for quality, selected egg meats were mixed in a sterile blender jar for 2 minutes at such a speed that foaming did not occur. The mixed whole egg was then strained through a double layer of sterile cheesecloth into a sterile beaker. This mixture was used in the thermal death time (TDT) tests.

If the egg was to be acidified, 10% lactic or 10% hydrochloric acid was added stepwise during the blending operation until the pH measured electrometrically on small aliquots reached the desired level.

Inoculation of the testing medium. A saline suspension of the organism was suitably diluted in sterile saline so that 1-5 ml. of the dilute suspension when added to a quantity (usually 200 ml.) of chilled liquid egg (or phosphate buffer solution) would provide a viable cell concentration of the desired magnitude. The mixture was then shaken for 200 cycles by hand or for 2 minutes on a mechanical shaking machine.

Filling the thermal death time (TDT) tubes. A sterile manually operated Cornwall Pipetting Unit adjusted to deliver 1 ml. at each stroke and fitted with a 4-inch stainless steel cannula was used to deliver the inoculated egg media into sterile standard TDT tubes *(8)*. Each tube received 2 ml. The filled TDT tubes were immediately sealed in an oxy-gas flame and held in a refrigerator until used, but not to exceed 2 hours.

Heating of the filled TDT tubes. Generally 5 racks, each containing 4 or 5 of the chilled tubes, were plunged simultaneously into the heated water bath maintained at a constant temperature, ± 0.2°F. Timing was begun at the moment of submersion. At each of five selected intervals of time, a rack was withdrawn from the bath and plunged into ice water.

Culturing of TDT tube contents. Two methods were used to incubate the treated cells. Method (a): The cooled tubes were scored with a file, dipped into 70% alcohol, flamed and opened. The tube contents were emptied by pouring into culture tubes containing 10 ml. of trypticase soy broth. These were incubated for 5 to 7 days at 37°C. and daily observations made for the presence of bacterial growth. When growth occurred the presence of *Salmonella* was confirmed by streaking a loopful of the tube contents on a section of a brilliant green agar (Difco) poured plate and incubating for 24 hours at 37°C. Method (b): The cooled tubes were placed in a 37°C. incubator and held for 5 days before opening and inoculation into the trypticase soy broth. This is the method used by Anellis *et al. (4)*. Subsequent steps were the same as for method (a).

Checking the cell load in test samples. One ml. quantities of the egg mixture or other media were delivered by the Cornwall pipetting unit directly into sterile saline blanks. A suitable dilution was made (1:1,000 when the count was approximately 100,000) and 1-ml. quantities were plated out in duplicate with trypticase soy agar. The plates were incubated at 37°C. for 24 hours and colony counts made.

Correction for heating lag. Correction for the heating lag in the TDT tubes was determined by the method of Sognefest and Benjamin *(24)*. Within the experimental tem-

perature range, 130°-142°F.(54.4°-61.1°C.), the correction factor to be subtracted from experimental heating time was found to be 0.9 minute for buffer solutions and 1.1 minutes for liquid egg media. These results fall closely within the range given by Ball, Merrill, Williams and Wessel (1937) (unpublished), as quoted by Sognefest and Benjamin *(24)* for TDT tubes in a water bath.

Estimation of heat resistance. The heat resistance was evaluated in terms of thermal death times (TDT) and/or decimal reduction times (D). The latter, which represents the time required to kill 90% of a population, was calculated from the relative numbers of positive and negative tubes, by using Schmidt's *(23,* also see *13)* method. The D values determined in this way as well as the TDT values are "end point" values and therefore place emphasis on any unusually resistant cells that may be present in the population. Both TDT and D values will be reported generally for 140°F.(60°C.). The symbols F_{140} will be used to indicate the TDT at 140°F. and z to indicate the reciprocal of the slope of the plot, log TDT *vs.* T°F. The two terms z and F define the thermal-death-time curve. The meaning of these factors, particularly z, is discussed by Rahn *(22)* in terms of Q_{10} and the thermodynamic temperature coefficient.

It is recognized that the death rate of vegetative microorganisms may not be exactly logarithmic *(15, 26)*. Nevertheless, the logarithmic law is most useful as an aid in presenting and interpolating data. The relation may be written:

$$\log (a/a-x) = kt/2.3 \tag{1}$$

where a is the initial count, x is number of organisms killed in time t, and k is constant for the culture and temperature used. Ideally, when $(a-x)$ becomes so small in terms of the probability that not one viable cell or growth unit occurs in any of 5 tubes (if it is a 5-tube run) then the corresponding t will equal TDT. Now if we start out with b cells instead of a, then a new TDT will be found when $(b-x)$ equals $(a-x)$, i.e., is so small that none of 5 tubes show growth. Hence, and since $k/2.3 = 1/D$ and substituting r for $(a-x)$ we may write

Obviously,

$$\log (a) = (TDT_a)/D + \log r \tag{2}$$

$$\log (a/b) = (TDT_a - TDT_b)/D \tag{3}$$

Equation (2) can be shown to be identical with the empirical equation reported by Gross, Vinton and Stumbo *(12)* to represent the relation between spore numbers and thermal death time. Amaha *(3)* reported another empirical equation representing the relation found in his studies. Use of equation (2) has the advantage that a plot of log (initial count) *vs.* TDT should give a line whose slope will be equal to 1/D. Obviously, the D obtained in this way will not necessarily be the same as the D obtained by only a 90% kill, since we are assuming a logarithmic death rate. This D value is an "end point" value and therefore should (and does in our experience) agree approximately with D values determined from the same data by the method of Schmidt *(23)*.

For comparative purposes TDT values can be calculated from D values whether or not they are obtained by "end point" procedures. To do this we simply use the relation given in equation (2) and estimate how low the number of organisms must be so that no tubes in a given set will show growth (probability basis). For a 5-tube run, r must be less than 0.22, since 1 tube out of 5 showing growth would indicate that the most probable number of organisms present is 0.22 per tube. A value of 0.1 for r leads to the relation $TDT/D = 1 + \log a$. Since for a 50-tube run the value of $\log r$ would be approximately 2, the exact value of r is not important except for very low values of a. The data of Gross *et al. (12)* indicate curiously a much lower value of the constant (in their equation) that corresponds to r in equation 2.

RESULTS

Effect of pH on heat resistance. The effect of pH on the heat resistance of *Salmonella* has been studied to a limited extent over the range pH 5.3 to 9.0, since pH 5.5 is the pH of the acidified type of stabilized whole egg and pH 9.0 is approximately the maximum pH of egg white. Table 1 shows that the F_{140} values for strains of *Salmonella* decrease as the pH is increased from pH 5.3 to 7 and from 7 to 8.2 when the cells

are heated in M/15 phosphate buffer. Anellis *et al. (4)* have made a careful study of the pH effect for *S. senftenberg* 775W in liquid whole egg and obtained results comparable to those reported here for heating in phosphate. Although the results show higher heat resistance of *Salmonella* at pH 5.3 than at pH 7 it is recognized, of course, that low pH values will kill *Salmonella* even without heat. Kintner and Mangel *(16)* found this to be true in a study of artificially infected salad dressing. Under their test conditions all of the 6 strains of *Salmonella* used were killed at pH 3.4 in 2 hours at room temperature. Four of the strains survived 24 hours at pH 4.56.

Relative F_{140} values in phosphate buffer (pH 7) and in whole egg, egg white, and egg yolk at their natural pH values. Table 2 shows for 8 strains of *Salmonella* that higher F_{140} values are obtained in liquid whole egg at pH 7.6 than in phosphate buffer at pH 7.0. As expected, medium as well as pH is a factor affecting the heat resistance. However, the low heat resistance of *Salmonella* in egg white at pH 9 is probably due to the high pH. Also, the higher heat resistance in yolk at pH 6.2 than in whole egg may be largely due to pH differences. These views are based on the observation that the differences in heat resistance are about what one would expect from the pH studies (*4*, and Table I). In any case, it is evident that it would take about twice as long to destroy *Salmonella* in egg yolk as in whole egg when both are at their natural pH.

TABLE 1

Effect of pH on F_{140} values of Salmonella suspended in phosphate buffer (Method a)

Strain		F_{140} values at following pH values[1]		
		5.3	7.0	8.2
		min.	*min.*	*min.*
S. meleagridis	5485	1.4	0.9	0.4
S. pullorum	3083	1.0	.7	.2
S. senftenberg	3252	1.4	.9	....
S. senftenberg	775W	16	7	1

[1]All F_{140} values reported in this and following tables are for approximately 200,000 organisms per tube unless otherwise stated. Each run consisted of 5 tubes.

Absence of relation between heat resistance and species and the occurrence of heat-resistant strains. Table 2 serves to illustrate that *Salmonella* strains are frequently similar in heat resistance. A notable exception is *S. senftenberg* 775W, which is highly heat resistant. Note also that *S. senftenberg* 3252 is of ordinary heat resistance. That is, it is like the non-senftenberg strains studied. *S. pullorum* 3083 is probably somewhat less heat resistant than the other strains. Anellis *et al. (4)* found that the strain of *S. pullorum* used in their studies had a low heat resistance. In addition to *S. pullorum* they studied *S. oranienburg* 1011, *S. montevideo*, *S. typhimurium* 81, and two

TABLE 2

Effect of medium (phosphate buffer, whole egg, egg white and egg yolk) on heat resistance of Salmonella (Method a)

Strain		Heat resistance (F_{140} values)[1]			
		Buffer pH 7.0	Whole egg pH 7.6	Egg white pH 9.0	Egg yolk pH 6.2
		min.	*min.*	*min.*	*min.*
S. anatis	5343	0.7	1.8	0.4	....
S. meleagridis	5485	.9	1.9	.4	4.4
S. newport	5761	.9	1.8	.6	....
S. pullorum	3083	.7	1.7	.3	3
S. senftenberg	3252	.9	1.9	.5	3.4
S. derby	5420	1.0	1.8	.5	....
S. bareilly	CM688	0.8	1.6	.6	....
S. senftenberg	775W	7	48	3.5	....

[1]See Table 1.

strains that we have studied, *S. meleagridis* 5485 and *S. senftenberg* 775W. Since *S. senftenberg* 775W is 10 to 20 times as heat resistant as other strains of *Salmonella* studied in this laboratory the question naturally arises, do such resistant organisms occur frequently? Solowey *et al. (25)* concluded that only 4 out of 95 cultures tested gave evidence of being more heat resistant than the other 91. Winter *et al. (31)* reported that some strains of *S. senftenberg* and *S. cerro* have high heat resistance. They worked with mixed strains, however, so that we are not sure how many strains were of high heat resistance. According to their results *S. typhimurium* (10 strains) is 1.8 times as heat-resistant as most other species, *S. cerro* is 2.6 times as heat-resistant, and *S. senftenberg* is 5.3 times as resistant. Estimates from the data of Solowey *et al. (25)* indicate that one strain, *S. senftenberg* 775W is 6.3 times as resistant as 91 other strains of *Salmonella* including 11 other strains of *S. senftenberg*. Under their conditions of test *S. cerro* was not found to be unusually heat-resistant. However, one strain of *S. oranienburg* and 2 of *S. paratyphi B*. were respectively approximately 4 and 5 times as heat resistant as the other strains. Winter *et al. (31)* tested 22 strains of *S. oranienburg* without finding unusual heat-resistance. They did not study *S. paratyphi B*. The one strain of *S. oranienburg* studied by Anellis *et al. (4)* was of "ordinary" heat-resistance. Beamer and Tanner *(5)* reported a strain of *S. paratyphi* to be less heat-resistant than *S. schottmuelleri* (ratio 2.1) or than *S. aertryke* (ratio 1.6).

The foregoing citations show that variations in heat resistance of *Salmonella* occur with respect to strain rather than with respect to species or group. The conflicting results could have arisen because of differences in strain, suspending medium, culturing method, count, etc. The unusually high heat resistance of *S. senftenberg* 775W has been confirmed in three separate laboratories, in one case by non-end point determinations of D and in the other two by end point determinations.

If heat-resistant strains of *Salmonella* occur to any great extent they would tend more than non-heat-resistant organisms to survive pasteurization and drying treatments. Therefore, the heat resistance of 10 isolates from pasteurized (and dried) egg was determined. Table 3 shows that these isolates were no more heat resistant than ordinary strains. Their presence in the powder may have resulted from improper operation of the pasteurizer, heavy contamination with strains of ordinary heat resistance, or recontamination after pasteurization or drying.

TABLE 3

Absence of heat-resistant strains among ten isolates from dried pasteurized [1] egg

Number and source of strains	D values in whole egg at 138°F. (Method a)	D values in egg yolk at 140°F. (Method b)
	min.	*min.*
4 isolates from whole egg	0.28 to 0.62	....
6 isolates from egg yolk	....	0.8 to 1.2
S. meleagridis (reference strain)	.43	.92

[1] The whole egg had been heated to 139°F. for at least 2 minutes; the egg yolk had been heated more than this but the exact conditions are not known.

Data presently available therefore indicate that heat-resistant strains are uncommon.

Effect of lactate at pH 5.5 on TDT. Liquid whole egg that had been acidified to pH 5.5 with lactic acid was used in several TDT runs. The TDT values obtained in the presence of the lactate (approx. 0.06 *M*) were from a half to a tenth the values obtained when hydrochloric acid was used to acidify the egg (Table 4).

It is well known that organic acids frequently have specific inhibitory effects on the growth of bacteria. Since the suspending medium has an effect on the heat resistance of microorganisms, it is not surprising that modification of the medium by addition of lactic acid decreases the heat resistance of *Salmonella* as compared with the addition of an amount of hydrochloric acid that would give the same pH. In contrast to lactic acid some substances, e.g. sugars, increase the heat resistance of *Salmonella (5)*. The possibility of improving the efficiency of pasteurization by use of appropriate additives is intriguing.

TABLE 4

Effect of lactic acid vs. hydrochloric acid on TDT values in liquid whole egg at pH 5.5

Strain	TDT values, F_{140}[1]	
	Lactic acid	HCl
	min.	*min.*
S. meleagridis		
Method a	2	5.1
Method b	3	6.2
S. senftenberg 775W		
Method b	9	95

[1] See Table 1.

Relation between numbers and TDT. The theoretical relation between numbers and TDT based on a logarithmic death rate has already been given (equation 3). The observed relation is given in Figure 1 for *S. meleagridis* and *S. senftenberg* 775W. The D values calculated by Schmidt's method averaged 0.35 for runs at 10^2 to 10^4 cells per tube and averaged 0.62 minute at the higher counts of *S. meleagridis*. D averaged 11.6 for *S. senftenberg*. The straight lines in the figure have been drawn to correspond to the D values found by the Schmidt method. It is evident that D values calculated by the Schmidt method and the values that would have been obtained by drawing a line from the TDT value to −1 on the ordinate are similar. However, under the conditions used here the observed values for *S. meleagridis* give a curve that is markedly concave downward and examination of the somewhat incomplete *S. senftenberg* data indicates the same trend. It would appear therefore that TDT values obtained at high counts will supply a *safe* margin (but not necessarily the *minimum* safe margin) by extrapolation to lower counts using −1 as the intercept, but not vice versa. With respect to the drawing of deductions from Figure 1 specifically, it must be noted that various factors such as pH, media, etc., may shift the position of the curves. Indeed, since the *S. senftenberg* data for the run represented by the superior struck circles indicates, for no known reason, a somewhat lower heat resistance than the data for the run represented by inferior struck circles, it is evident

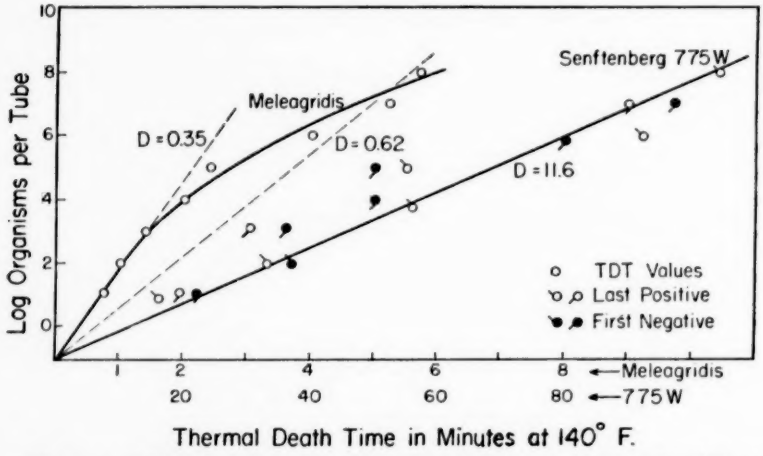


Figure 1. Relation between count and thermal death times for S. meleagridis and S. senftenberg 775 W (method b). For S. senftenberg the upper struck and the lower struck circles respectively indicate runs made on different days but on aliquots of the same cell suspension.

that variations from run to run are to be expected, as we have found to be true to some extent in other cases.

The data in Tables 5a and 5b show the relation between numbers of organisms and the ratio of TDT to D. The ratio should theoretically be independent of medium, pH, temperature, and the value of D (the heat resistance). It should depend and apparently does depend on numbers only. The observed D values for the ratios reported varied over 30-fold. Note that we have used "end point" D values calculated by the Schmidt method. In our opinion TDT values calculated (equation 2) from D values based on all sets of tubes that are partly negative are more accurate than TDT values estimated only from the heating times that bracket sets of tubes showing no growth

TABLE 5a

Comparison of observed and calculated ratios of TDT and D for widely varying numbers of cells

Number of organisms	TDT/D		
	Calculated ratio = $1 + \log a$	Observed	
		S. meleagridis[1]	*S. senftenberg*[1] 775W
10	2	....	2.1
100	3	3.0	2.5
1,000	4	4.1	4.5
10,000	5	5.0	....
100,000	6	6.4	6.0
1,000,000	7	8.0	....
10,000,000	8	9.1	8.0
100,000,000	9	9.2	....

[1]Organisms were heated in whole egg at pH 7.5.

and some growth. The former procedure also minimizes the problem of "skips." However, for the purposes of this paper we have reported TDT values estimated in the usual manner.

Effect of method (or medium used to culture heated organisms) on apparent heat resistance. The procedure of transferring heated whole egg and yolk samples to trypticase soy broth as soon as they are cool has consistently yielded lower TDT and D values than the procedure of incubating the samples in the egg meat prior to transferring. Table 6 gives both the D values and ratio of the D values obtained by the two methods of incubation. Method (b) gives D values averaging nearly 50% higher than the values obtained by method (a). The standard error of the mean ratio indicates that this increase is significant at a high confidence level.

The results indicate that whole egg (and yolk) is a better medium for growing small numbers of cells than trypticase soy broth. However, this does not appear to be

TABLE 5b

Comparison of observed and calculated ratios of TDT and D under various conditions

Strains	Medium	pH	TDT/D	
			Calculated	Observed
S. newport (5761)	WE[1]	7.6	6.3	7.2
S. pullorum (3083)	Yolk	6.2	6.0	5.5
S. meleagridis (5485)	Yolk	6.2	6.4	5.9
S. montevideo (29G/200E)	Yolk	6.2	6.5	6.7
S. pullorum (3083)	White	5.5	5.9	5.2
S. meleagridis (5485)	White	5.5	6.2	6.0
S. meleagridis (5485)	White	8.9	6.2	6.0
S. meleagridis (5485)	WE	7.6	4.3	4.6
S. anatis (5343)	WE	7.6	6.3	6.3

[1]WE = whole egg.

the case when the cells had not been subjected to heat treatment. Table 7 shows that unheated cells grow in trypticase soy broth, if anything, slightly better than in whole egg. The simplest interpretation of these observations is that heated cells (damaged cells) recover better in egg than in broth even though broth is just as good a medium for undamaged cells. This is similar to results of Curran and Evans *(6)*, Amaha *(1, 2, 3)* and others (cf. ref. *5)*, who show heated or damaged cells are much more exacting in their nutritional requirements. Furthermore, temperature of incubation following heating affects the apparent TDT of *Clostridium botulinum (28)*. This is another factor

TABLE 6

Effect of recovery media following heat treatment on observed D values (S. meleagridis)

Suspending media during heating	D values at 138°F.		Ratio: D (b) / D (a)
	Method (a)—trypticase soy	Method (b)—whole egg or yolk	
	min.	*min.*	
Whole egg pH 7.6	.43	.53	1.23
Whole egg pH 7.6	.42	.78	1.86
Whole egg pH 7.6	.43	.74	1.72
Whole egg pH 5.5	1.15	1.53	1.33
Whole egg pH 5.5	.90	1.27	1.41
Whole egg pH 5.5	.84	1.39	1.65
Whole egg pH 5.5	.47[1]	.51	1.08
Whole egg pH 5.5	.49[1]	.60	1.22
Whole egg pH 5.5	.37[1]	.63	1.70
Egg yolk pH 6.3	.84	1.37	1.63
		Mean	1.48
		Standard error	0.083
		Standard deviation	0.26

[1]These three runs were made with a culture that was less resistant to heat than usual (no reason could be found for this).

TABLE 7

Comparison of trypticase soy broth and whole egg as media for growing out small numbers of unheated cells (S. meleagridis)

Positive tubes in 10-tube runs at various dilutions			
Test A		Test B	
TSB	WE	TSB	WE
10	9	10	10
8	6	9	9
6	7	7	3
1	3	2	2
3	1		

that may be of importance in the case of *Salmonella* TDT's and is possibly another reason why conflicting results are obtained in different laboratories.

The possibility that mechanical factors or dilution factors might cause the differences observed with the two methods has been minimized by comparing TDT and D values obtained by: (a) incubating directly in the TDT tubes, (b) transferring the tube contents to new TDT tubes, (c) transferring the tube contents to "sterile" whole egg, and (d) transferring the tube contents to trypticase soy broth. The results (Table 8) show that only transfers to trypticase soy broth reduce the TDT and D values.

TABLE 8

Effect of incubating heated cells in whole egg and in trypticase soy broth on TDT and D values (138°F.)

Incubation method following heat treatment	TDT[1]	D[1]
	min.	*min.*
Whole egg in original TDT tubes	4.0	.58
Transfer to new TDT tubes	3.7	.56
Transfer to sterile whole egg	3.9	.56
Transfer to TS broth	2.8	.44

[1]Average of three 5-tube runs in which all four incubation methods were run side by side.

DISCUSSION

The answer to the question whether a reliable practical pasteurization procedure for whole egg is possible depends obviously on the relative heat-lability of both the egg and *Salmonella*.

It is generally believed that it is not practical to heat whole egg above 143°F. for more than about four minutes [cf. Goresline *et al. (11)*, who recommend even a slightly milder treatment]. This is true more because of technical difficulties (excessive coagulation of egg on heating surfaces) than because of changes in functional properties of the egg. Thus, there is at best a narrow margin between the heat treatment required to destroy *Salmonella* and a heat treatment that will be practical with respect to coagulation and film formation on the heating plates. It is for this reason that we have made a careful study of *Salmonella* heat resistance. Thus, if *Salmonella* is sufficiently heat-sensitive under some otherwise suitable condition, then pasteurization might still be accomplished with a good degree of assurance that a safe product is being produced. It must be remembered that pasteurization is not a cure-all nor is it fool proof *(7)*. It is never a substitute for good sanitation and the pasteurized product must be handled with judgment to prevent recontamination.

It is instructive to make more or less reasonable assumptions in order to see what might be expected from pasteurization treatments. Thus, let us use pH 7.5 whole egg; let us consider "ordinary" strains and the heat-resistant *S. senftenberg* 775W separately, and let us assume we want to reduce the *Salmonella* count to less than 1 in 100 g. If we use an F_{140} value of 6 strains of "ordinary" heat resistance and an F_{140} value of 70 for *S. senftenberg* 775W and make suitable calculations (see footnote in Table 9) we obtain the values shown in Table 9. The table emphasizes the importance of initial numbers of *Salmonella* as well as time and temperature.

Obviously, if the numbers can be kept low, say less than 1,000 per g. in the raw product, heat treatment for 3 minutes at 142°F. would give a reduction in count to less than one *Salmonella* per 100 g. so far as *Salmonella* of "ordinary" heat resistance is concerned. For *S. senftenberg* 775W, and similar organisms if they occur, we could not tolerate more than one organism in 40 g. Can such low counts be obtained by shell sanitation and highly sanitary breaking facilities and operations? Even though highly heat-resistant strains may be uncommon, as has been indicated in this paper, nevertheless these and other heat-resistance studies

show that every practical effort should be made to achieve, in practice, a very low *Salmonella* count prior to pasteurization.

In liquid egg yolk, *Salmonella* organisms are about twice as stable to heat as in whole egg (apparently because the pH is lower). Therefore, about twice the times shown in Table 9 would be needed to pasteurize liquid yolk. Higher temperatures, if permissible from other considerations, would of course reduce the times required.

TABLE 9

Calculated times required to reduce Salmonella counts to less than 1 in 100 grams of liquid whole egg under more or less reasonable assumptions[1]

Count: Number per g.	Pasteurization times at following temperatures for strains of "ordinary" heat resistance		
	140°	142°	144°
	min.	*min.*	*min.*
10	2.9	1.7	1.0
10,000	5.7	3.4	2.0
1,000,000	7.6	4.6	2.7
	For S. senftenberg 775W		
0.025	4.4	2.6	1.6
0.1	11	6.6	4
10	33	19	12
10,000	66	40	24
1,000,000	88	53	32

[1]The values in this table were calculated in the following way: The value $F_{140} = 6$ was used for *Salmonella* strains of "ordinary" heat resistance (about twice the value reported in Figure 1 for *S. meleagridis* has been used intentionally). Whereas it appears from Table 2 that there are only very small differences in heat resistance among "ordinary" strains, this evidently is not always the case (*4, 5, 23, 31*). The value $F_{140} = 70$ was used for *S. senftenberg* 775W (Figure 1). Unreported data yielded values of approximately 9 for z and this value has been used to calculate the TDT values at 142° and 144°F. using the relation $\log (D_2/D_1) = \theta_1 - \theta_2/z$ where θ_1 and θ_2 are the temperatures corresponding to D_1 and D_2. Now, if $F_{140} = 6$ for 200,000 cells per tube, then $D = TDT/\log (2 \times 10^5/0.1)$ (c.f. eq. 2 and discussion of the value of r) or $D = 6/6.3 = 0.95$; hence $\log (a) = TDT/0.95 + \log (0.01)$ (c.f. eq. 2) so that TDT can be calculated for any value of a, where a corresponds to the count per gram initially and 0.01 corresponds to the final count per gram. A value of 0.01 means virtually that no growth would occur in any tubes of a 5-tube run where each tube (or flask) contained 20 g. of sample. Since $D_{142} = D_{140} \times$ antilog $(140-142)/9$ the 142° TDT values can be calculated readily for the various counts.

In liquid egg white, one-third to one-tenth the time would be required to kill *Salmonella* (apparently because the pH is high). However, egg white is so sensitive to heat that it may not be possible to devise an entirely satisfactory commercial pasteurization procedure for egg white. Certainly it would require the use of lower temperatures than 140°F., as pointed out by previous workers (*27, 29*).

Although keeping numbers low prior to pasteurization is of fundamental importance, it is pertinent to discuss the significance of pH and additives such as lactic acid (which is permissible in some products) with respect to practical pasteurization. Thus, in the neighborhood of pH 9 much less time (one-third or less) than that reported in Table 9 would be required to give a kill equivalent to that found at pH 7.5. Beamer and Tanner (*5*) showed that *Salmonella* organisms heated to 140°F. are killed several-fold more quickly in tomato juice at pH 4.2 than in broth at pH 7.05; they are killed still more quickly in broth at pH 3.65. Also, they die rapidly at room temperature in salad dressing when the pH is below 4.0 (*15*). Obviously, then, it may be possible to affect some control by use

of low pH treatments (with or without heat) even in products that do not remain at the acid pH. Some control might be affected also by the use of such substances as lactic and acetic acids [the latter was present in the salad dressing *(15)*] in combination with mild heat treatment of products such as egg white in cases where such additions are permissible.

In conclusion the data establish in a quantitative way certain aspects of egg pasteurization: (a) It is imperative to keep the initial count of *Salmonella* as low as possible in order to reduce the chance that some *Salmonella* organisms will survive pasteurization; that is, the greater the number of organisms present, the greater the time required to reduce their number to any given value. (b) It may be well worthwhile to examine means of enhancing the efficiency of killing without increasing the coagulation, thickening or film deposition of egg meats on heating plates. The means that are suggested from the present study are pH adjustment and use of an additive [pasteurization accelerator *(17)*], such as lactic acid where permissible. Further studies are needed to evaluate both the factors that influence the initial *Salmonella* counts in egg products and the precise conditions under which it is practical to enhance the pasteurization efficiency.

SUMMARY AND CONCLUSIONS

The heat resistance of 18 strains of *Salmonella* have been described in terms of decimal reduction times (D) and thermal death times (TDT). Representative strains of several species were used to assess the influence of pH, media (egg yolk, egg white, whole egg, and buffer), numbers, and method of recovery on the D and TDT.

Heat resistance varied with strain rather than with species or group. Heat resistance is greater near pH 5.5 than above pH 7. Liquid whole egg is a better medium for growth of heat damaged cells than trypticase soy broth. The observed relation between numbers and TDT approximated the theoretical relation at lower counts, but not at higher counts. The TDT was found to be equal approximately to D, calculated by Schmidt's method, multiplied by the sum 1 + log (initial count), in agreement with theory for 5-tube runs.

The heat-resistant strain, *S. senftenberg* 775W, is an important stumbling block in the path of successful egg pasteurization. Even though heat-resistant strains are not frequently encountered, the need is emphasized for keeping *Salmonella* counts very low (less than 10 per 100 g.) in order to obtain, with good assurance, a pasteurized product containing less than one *Salmonella* per 100 g. This conclusion is based on the fact that unmodified whole egg, for example, cannot be heated more than several minutes at 143°F. without coagulation and film formation on the heating plates.

Acknowledgment

We wish to express our appreciation to the Quartermaster Food and Container Institute for the Armed Forces for permitting one of us (William W. Osborne) to conduct comparative tests in the Institute laboratories over a three weeks' period and to Dr. Morton Rayman and Mr. Abe Anellis of the Institute for their cooperation at this time and also for helpful discussions. We are indebted to Dr. Rayman of the Institute and to Dr. Glen Slocum of the Food and Drug Administration for the *Salmonella* isolates obtained from heat-treated (pasteurized) egg solids.

LITERATURE CITED

1. AMAHA, M. Studies on the heat resistance of bacterial spores. Part 5. Influences of vitamins and amino acids in the synthetic subculture media on the survival times. *J. Agr. Chem. Soc. Japan*, **26**, 420 (1952).
2. AMAHA, M. Studies on the heat resistance of bacterial spores. Part 3. The effects of sugars in the subculture media on the survival times of *Bacillus matto*. *J. Agr. Chem. Soc. Japan*, **26**, 306 (1952).
3. AMAHA, M. Heat resistance of Cameron's putrefactive anaerobe 3679 in phosphate buffer *(Clostridium sporogenes)*. *Food Research*, **18**, 411 (1953).
4. ANELLIS, A., LUBAR, J., AND RAYMAN, M. M. Heat resistance in liquid eggs of some strains of the genus *Salmonella*. *Food Research* (this issue).
5. BEAMER, P. R., AND TANNER, F. W. Resistance of non-spore-forming bacteria to heat. *Zentr. Bakt. Parasitenk.* Abt. II, **100**, 81 (1939).
6. CURRAN, H. R., AND EVANS, F. R. The importance of enrichment in the cultivation of bacterial spores previously exposed to lethal agencies. *J. Bact.*, **34**, 179 (1937).
7. ECKLES, C. H., COMBS, W. B., AND MACY, H. *Milk and Milk Products*. 3rd ed., 1943. McGraw-Hill Book Company, Inc., N. Y.
8. ESTY, J. R., AND WILLIAMS, C. C. Heat resistance studies. I. A new method for the determination of heat resistance of bacterial spores. *J. Infectious Diseases*, **34**, 516 (1924).
9. GIBBONS, N. E., FULTON, C. O., AND REID, M. Dried whole egg powder. XXI. Pasteurization of liquid egg and its effect on quality of the powder. *Can. J. Res., F.*, **24**, 327 (1946).
10. GIBBONS, N. E., AND MOORE, R. L. Dried whole egg powder. XI. Occurrence and distribution of *Salmonella* organisms in Canadian powder. *Can. J. Res., F.*, **22**, 48 (1944).
11. GORESLINE, H. E., HAYES, K. M., MOSER, R. E., HOWE, M. A., AND DREWNIAK, E. E. Pasteurization of liquid whole egg under commercial conditions to eliminate *Salmonella*. U. S. Department of Agriculture *Circular No. 897*.
12. GROSS, C. E., VINTON, C., AND STUMBO, C. R. Bacteriological studies relating to thermal processing of canned meat. VI. Thermal death-time curve for spores of test putrefactive anaerobe in meat. *Food Research*, **11**, 411 (1946).
13. HALVORSON, H. O., AND ZIEGLER, N. R. Application of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. *J. Bact.*, **25**, 101 (1933).
14. HINSHAW, W. R., AND MCNEIL, E. *Salmonella* infection as a food industry problem. *Advances in Food Research*, **3**, 209 (1951). Academic Press, Inc., N. Y.
15. HINSHELWOOD, C. Decline and death of bacterial populations. *Nature*, **167**, 666 (1951).
16. KINTNER, T. C., AND MANGEL, M. Survival of staphylococci and salmonellae experimentally inoculated into salad dressing prepared with dried eggs. *Food Research*, **18**, 6 (1953).
17. LEWIS, J. C., MICHENER, H. D., STUMBO, C. R., AND TITUS, D. D. Antibiotics in the preservation of processed foods. *J. Agri. Food Chem.* (in press).
18. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. I. Pathogenicity of strains of *Salmonella meleagridis* and *Salmonella anatum* obtained from spray-dried whole egg. *J. Infectious Diseases*, **88**, 278 (1951).
19. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. II. Immunity studies following experimental illness with *Salmonella meleagridis* and *Salmonella anatum*. *J. Immunol.*, **66**, 595 (1951).
20. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. III. Pathogenicity of strains of *Salmonella newport*, *S. derby*, and *S. bareilly* obtained from spray-dried whole egg. *J. Infectious Diseases*, **89**, 209 (1951).
21. MCCULLOUGH, N. B., AND EISELE, C. W. Experimental human salmonellosis. IV. Pathogenicity of strains of *Salmonella pullorum* obtained from spray-dried whole egg. *J. Infectious Diseases*, **89**, 259 (1951).
22. RAHN, O. Physical methods of sterilization of microörganisms. *Bact. Rev.*, **9**, 1 (1945).

23. SCHMIDT, C. F. Thermal resistance of microorganims. Page 732 of Chapter 32 in *Antiseptics, Disinfectants, Fungicides and Sterilization* (G. F. Reddish, Ed.), Lea and Febiger, Philadelphia, 1954.
24. SOGNEFEST, P., AND BENJAMIN, H. A. Heating lag in thermal death-time cans and tubes. *Food Research*, **9**, 234 (1944).
25. SOLOWEY, M. SUTTON, R. R., AND CALESNICK, E. J. Heat resistance of *Salmonella* organisms isolated from spray-dried whole-egg powder. *Food Technol.*, **2**, 9 (1948).
26. WHITE, R. H. The heat resistance of *Streptococcus faccalis*. *J. Gen. Microbiol.* **8**, 27 (1953).
27. WILKIN, M., AND WINTER, A. R. Pasteurization of egg yolk and white. *Poultry Sci.*, **26**, 136 (1947).
28. WILLIAMS, O. B., AND REED, J. M. The significance of the incubation temperature of recovery cultures in determining spore resistance to heat. *J. Infectious Diseases*, **71**, 225 (1942).
29. WINTER, A. R. Production of pasteurized frozen egg products. *Food Technol.*, **6**, 414 (1952).
30. WINTER, A. R., GRECO, P. A., AND STEWART, G. F. Pasteurization of liquid-egg products. I. Bacteria reduction in liquid whole egg and improvement in keeping quality. *Food Research*, **11**, 229 (1946).
31. WINTER, A. R., STEWART, G. F., MCFARLANE, V. H., AND SOLOWEY, M. Pasteurization of liquid egg products. III. Destruction of *Salmonella* in liquid whole egg. *Am. J. Pub. Health*, **36**, 451 (1946).

DIFCO

Bacto-Dehydrated Media for Microbiological Assay of
VITAMINS and AMINO ACIDS

These media contain all the necessary nutriments for the growth of specified test organisms for the microbiological assay of vitamins and amino acids except for the component under assay. These basal media require only the addition of specified increasing amounts of the vitamin or amino acid being assayed to obtain a growth response which may be measured by acidimetric or turbidimetric methods for the construction of standard curves. The vitamin or amino acid content of the material under assay is determined by adding appropriate concentrations of the test substance to the basal medium and comparing the growth response obtained with that of the standard.

BACTO-RIBOFLAVIN ASSAY MEDIUM
BACTO-NIACIN ASSAY MEDIUM
BACTO-THIAMIN ASSAY MEDIUM
BACTO-PANTOTHENATE ASSAY MEDIUM
BACTO-B_{12} ASSAY MEDIUM USP
BACTO-CS VITAMIN B_{12} AGAR
BACTO-FOLIC ACID ASSAY MEDIUM
BACTO-PYRIDOXINE ASSAY MEDIUM
BACTO-BIOTIN ASSAY MEDIUM
BACTO-CHOLINE ASSAY MEDIUM
BACTO-CF ASSAY MEDIUM
BACTO-TRYPTOPHANE ASSAY MEDIUM
BACTO-LEUCINE ASSAY MEDIUM
BACTO-METHIONINE ASSAY MEDIUM
BACTO-LYSINE ASSAY MEDIUM
BACTO-ISOLEUCINE ASSAY MEDIUM
BACTO-ARGININE ASSAY MEDIUM
BACTO-TYROSINE ASSAY MEDIUM
BACTO-CYSTINE ASSAY MEDIUM

The method employed in carrying stock cultures of the test organisms and preparing the inoculum for microbiological assay is important. The following media have been developed especially for carrying stock cultures and for preparation of the inoculum:

BACTO-MICRO ASSAY CULTURE AGAR
BACTO-MICRO INOCULUM BROTH
BACTO-B_{12} CULTURE AGAR USP
BACTO-B_{12} INOCULUM BROTH USP
BACTO-NEUROSPORA CULTURE AGAR

BACTO-VITAMIN FREE CASAMINO ACIDS, dehydrated, is an acid hydrolysate of vitamin free casein prepared especially for laboratories investigating microbiological assay of vitamins.

Descriptive literature available upon request

DIFCO LABORATORIES
DETROIT 1, MICHIGAN